องค์ประกอบทางเคมีของรากมะควัด *Zizyphus rugosa* Lam. และรากมะเม่าสร้อย *Antidesm acidum* Retz.

นายสุทิน แก่นนาคำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2554

CHEMICAL CONSTITUENTS FROM ROOTS OF *Zizyphus rugosa* Lam. AND ROOTS OF *Antidesma acidum* Retz.

Mr. Sutin Kaennakam

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
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Thesis Title	CHEMICAL CONSTITUENTS FROM ROOTS OF *Zizyphus rugosa* Lam. AND ROOTS OF *Antidesma acidum* Retz.
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Field of Study	Chemistry
Thesis Advisor	Associate Professor Santi Tip-pyang, Ph.D.

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สุทิน แก่นนาคำ : องค์ประกอบทางเคมีของรากมะควัด *Zizyphus rugosa* Lam. และรากมะเม่าสร้อย *Antidesm acidum* Retz. (CHEMICAL CONSTITUENTS FROM ROOTS OF *Zizyphus rugosa* Lam. AND ROOTS OF *Antidesma acidum* Retz.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. สันติ ทิพยางค์, 83 หน้า.

การศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพจากสิ่งสกัดไดคลอโรมีเทนและเมทานอลของรากมะควัด *Zizyphus rugosa* Lam. สามารถแยกสารในกลุ่มไตรเทอร์พีนอยด์ได้ 9 ชนิด คือ lupeol (**1.1**), betulin (**1.2**), betulinic aldehyde (**1.3**), betulinic acid (**1.4**), alphitolic acid (**1.5**), euscaphic acid (**1.6**), zizyberenalic acid (**1.7**) และของผสมระหว่าง β-sitosterol (**1.8**) และ stigmasterol (**1.9**) สารในกลุ่มคูมาริน 1 ชนิด คือ scopoletin (**1.10**) สารในกลุ่มฟลาโวนอยด์ 4 ชนิด คือ kaempferol (**1.11**), afzelin (**1.12**), quercitrin (**1.13**) และ (+)-catechin (**1.14**) การพิสูจน์โครงสร้างของสารทั้งหมดที่แยกได้นี้ อาศัยวิธีทางกายภาพและวิธีทางสเปกโทรสโกปี ร่วมกับการเปรียบเทียบกับข้อมูลที่มีรายงานแล้ว จากการทดสอบฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด KB และ HeLa พบว่าสาร **1.2** และ **1.7** มีฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด KB และ HeLa ได้ปานกลาง โดยมีค่า IC_{50} เท่ากับ 10.0, 5.5 µg/mL และ 9.5, 13.0 µg/mL ตามลำดับ และสาร **1.4** มีฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด HeLa ได้ปานกลาง โดยมีค่า IC_{50} เท่ากับ 10.0 µg/mL

การศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพจากสิ่งสกัดไดคลอโรมีเทนและเมทานอลของรากมะเม่าสร้อย *Antidesma acidum* Retz. สามารถแยกสารใหม่จากธรรมชาติที่มีรายงานการสังเคราะห์มาแล้ว 1 ชนิด คือ corylifolin (**2.2**) พร้อมกับสารที่มีรายงานแล้วอีก 16 ชนิด คือ antidesmol (**2.1**), mellein (**2.3**), ของผสมระหว่าง β-stitosterol (**2.4**) และ stigmasterol (**2.5**), 5-cholesten-3 β-ol (**2.6**), 4-cholesten-3-one (**2.7**), 3-(1,1-dimethylallyl)-scopoletin (**2.8**), 5,7-dihydroxy-2-eicosyl-chromone (**2.9**), 2,5-dimethoxy-1,4-bezoquinone (**10**), barbatumol A (**2.11**), *N-trans*-feruloyltyramine (**2.12**), syringic aldehyde (**2.13**), *p*-hydroxybenzoic acid (**2.14**), taxifolin (**2.15**), (+)-catechin (**2.16**) และ (-)-gallocatechin (**2.17**) การพิสูจน์โครงสร้างของสารทั้งหมดที่แยกได้นี้ อาศัยวิธีทางกายภาพและวิธีทางสเปกโทรสโกปี ร่วมกับการเปรียบเทียบกับข้อมูลที่มีรายงานแล้ว จากนั้นนำสารทั้งหมดที่แยกได้ไปทดสอบฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด KB และ HeLa พบว่า สาร **2.9** และ **2.10** มีฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด HeLa ได้ดี โดยมีค่า IC_{50} ของเซลล์มะเร็งชนิด HeLa เท่ากับ 3.9 และ 1.6 µg/mL และพบว่าสาร **2.12**, **2.14** และ **2.17** ก็มีฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด HeLa ได้ปานกลางโดยมีค่า IC_{50} ของเซลล์มะเร็งชนิด HeLa เท่ากับ 12.3, 10.8 และ 14.9 µg/mL ในขณะที่สาร **2.10** และ **2.12** ยังมีฤทธิ์ในการยับยั้งเซลล์มะเร็งชนิด KB ได้ปานกลางโดยมีค่า IC_{50} ของเซลล์มะเร็งชนิด KB เท่ากับ 4.9 และ 7.8 µg/mL นอกจากนี้ยังนำสารที่แยกได้ไปทำการทดสอบหาฤทธิ์การยับยั้งเอนไซม์แอซีทิล และบิวทิริล โคลีนเอสเทอเรส ด้วยวิธีของ Ellman ซึ่งเป็นวิธีทางสเปกโทรสโกปี พบว่า สาร **2.2**, **2.3** และ **2.12** มีฤทธิ์ในการยับยั้งเฉพาะบิวทิริล โคลีนเอสเทอเรส ได้ต่ำที่ค่าเปอร์เซ็นต์การยับยั้งเท่ากับ 47.78, 40.29 และ 43.62 ตามลำดับ

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 .....

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KEYWORDS: *Zizyphus rugosa* Lam. / *Antidesma acidum* Retz.

SUTIN KAENNAKAM: CHEMICAL CONSTITUENTS FROM ROOTS OF *Zizyphus rugosa* Lam. AND ROOTS OF *Antidesma acidum* Retz.
ADVISOR: ASSOC. PROF. SANTI TIP-PYANG, Ph.D., 83 pp.

The investigation for chemical constituents from the CH_2Cl_2 and MeOH crude extracts of the roots of *Zizyphus rugosa* Lam. led to the isolation of nine triterpeniods; lupeol (**1.1**), betulin (**1.2**), betulinic aldehyde (**1.3**), betulinic acid (**1.4**), alphitolic acid (**1.5**), euscaphic acid (**1.6**), zizyberenalic acid (**1.7**), a mixure of *β*-sitosterol (**1.8**) and stigmasterol (**1.9**), one coumarin; scopoletin (**1.10**) and four flavonoids; kaempferol (**1.11**), afzelin (**1.12**), quercitrin (**1.13**) and (+)-catechin (**1.14**). The chemical structures of all isolated compounds were characterized according to physical means of spectral analysis as well as comparison with the previous literature data. All of the isolated compounds (**1.1**-**1.14**) were evaluated for their cytotoxicity on KB and HeLa cells. Compounds **1.2** and **1.7** exhibited moderate cytotoxicity against both KB and HeLa cells, with IC_{50} values of 10.0, 5.5 μg/mL and 9.5, 13.0 μg/mL, respectively, while compound **1.4** showed only moderate cytotoxic against on HeLa cells (IC_{50} = 10.0 μg/mL).

The phytochemical investigation of the CH_2Cl_2 and MeOH crude extracts from the roots of *A.acidum* Retz. afforded a new natural (synthetically known) compound, corylifolin (**2.2**), togather with sixteen compounds including antidesmol (**2.1**), mellein (**2.3**), a mixture of *β*–stitosterol (**2.4**) and stigmasterol (**2.5**), 5-cholesten-3*β*-ol (**2.6**) and 4-cholesten-3-one (**2.7**), 3-(1,1-dimethylallyl)-scopoletin (**2.8**), 5,7-dihydroxy-2-eicosyl-chromone (**2.9**), 2,5-dimethoxy-1,4-bezoquinone (**2.10**), barbatumol A (**2.11**), *N*-*trans*-feruloyltyramine (**2.12**), syringic aldehyde (**2.13**), *p*-hydroxybenzoic acid (**2.14**), taxifolin (**2.15**), (+)-catechin (**2.16**) and (-)-gallocatechin (**2.17**). Compounds **2.9** and **2.10** showed only good cytotoxicity against on HeLa cells (with IC_{50} values of 3.9 and 1.6 μg/mL) and compounds **2.12**, **2.14** and **2.17** showed only moderate cytotoxicity against on HeLa cells (with IC_{50} values of 12.3, 10.8 and 14.9 μg/mL), while compounds **2.10** and **2.12** showed only moderate cytotoxicity against on KB cells (with IC_{50} values of 4.9 and 7.8 μg/mL). The investigation and evaluation for the AChE and BuChE inhibitory activity using Ellman colorimetric method indicated that compounds **2.2**, **2.3** and **2.12** showed low inhibitory activity toward only BuChE with percentage inhibitory activity values of 47.78, 40.29 and 43.62, respectively.

Department : Chemistry Student's Signature : .....
Field of Study : Chemistry Advisor's Signature : .....
Academic Year : 2011

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My appreciation is also expressed to thank Dr. Pongpan Siripong and the staffs of Natural Products Research Section, Research Division, National Cancer Institute for testing on cytotoxicity against KB and HeLa cell lines experiment and Assistant Professor Dr. Pattra Sawasdee for testing on the AChE and BuChE inhibitory activity.

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LIST OF ABBREVIATIONS

^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
brs	broad singlet (NMR)
COSY	correlated spectroscopy
d	doublet (NMR)
dd	doublet of doublet (NMR)
ESIMS	electrospray ionization mass spectrometry
g	gram (s)
HMBC	heteronuclear multiple bond correlation
HRESIMS	high resolution electrospray ionization mass spectrometry
HSQC	heteronuclear single quantum correlation
Hz	hertz
IC_{50}	concentration that is required for 50% inhibition in vitro
J	coupling constant
m	multiplet (NMR)
M	molar
MeOH	methanol
mg	milligram (s)
MHz	megahertz
min	minute
mL	milliliter (s)
NMR	nuclear magnetic resonance
q	quartet (NMR)
s	singlet (NMR)
t	triplet (NMR)
UV	ultraviolet
VLC	vacuum liquid chromatography

δ	chemical shift
δ_C	chemical shift of carbon
δ_H	chemical shift of proton
μ	micro

CHAPTER I
CHEMICAL CONSTITUENTS FROM ROOTS OF *Zizyphus rugosa* Lam.

1.1 Introduction

Some tropical plants have many potential bioactive activities to be used in antithrombotic, antiinflammatory and antimutagenic activities. In addition, some of them also have a great potential anticancer activity in the prevention of cancers. In present times, plant based drugs provide outstanding contribution to modern therapeutics and have been investigated on wide diversity for conventional drug development.

In the present time, accident, HIV, cardiovascular and cancer were the leading causes of death in Thailand. The mortality rates from cancers have increased and became crisis of the world. Thus, scientists are still trying to find the treatment and the prevention that is the most potential to anticancer and the management of infectious and chronic diseases. Several natural compounds are endowed with potent anticancer activity for overcoming cancer cell resistance to chemotherapy [1].

Plants synthesized a wide variety of primary and secondary metabolites that toxic to plant predators. In addition, the secondary metabolites are highly varied in structure and have beneficial effects when used to treat human diseases such as phenols or their oxygen-substituted on aromatic compounds. Many spices of the medicinal plants yield useful medicinal compounds [2].

Researchers classified more than 122 compounds used in mainstream medicine which were derived from "ethnomedical" plant sources. 80% of these compounds were used as same as the traditional ethnomedical use. Traditional use of medicines is recognized as a way to learn about potential future medicines [3].

1.1.1 Triterpene biosynthesis pathway

Several triterpenes have been isolated from *Zizyphus* (Rhamnaceae), which are the characteristic of secondary metabolites of plants in this genus.

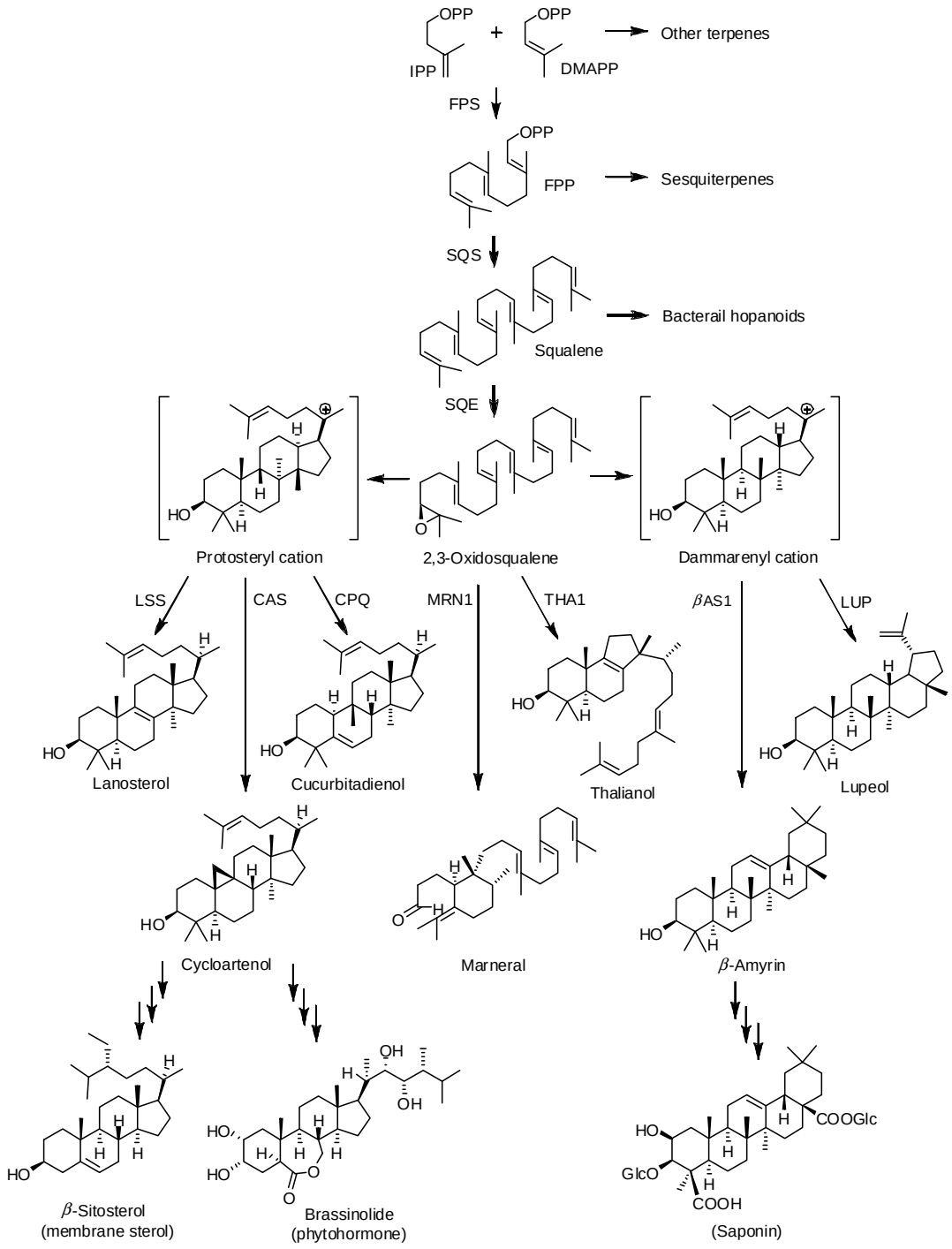


Figure 1.1 Biosynthetic pathways of triterpenes.

From the scheme of triterpene biosynthesis, farnesyl diphosphate synthase (FPS) isomerizes isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to farnesyl diphosphate (FPP), which squalene synthase (SQS) converts to squalene. Squalene epoxidase (SQE) oxidizes squalene to 2,3-oxidosqualene. OSC enzymes cyclize 2,3-oxidosqualene through cationic intermediates to triterpene alcohols or aldehydes. OSC products can be further modified by multiple enzymes to form membrane sterols, brassinosteroids, saponins, and other compounds. βAS1, β-amyrin synthase; LUP, lupeol synthase; MRN1, marneral synthase; PP, diphosphate; THA1, thalianol synthase (Figure 1.1) [4].

1.1.2 Chemical constituents from *Zizyphus rugosa* and their biological activities

Ziziphus rugosa have been used as a traditional medicine for the treatment of diarrhea, menorrhagia and infection of teeth [5]. The MeOH, CH_2Cl_2 and hexane extracts of its bark and leaves also showed antibacterial activity [6]. There are several triterpenes, flavonoids and alkaloids were isolated from many parts from this plant.

1.1.2.1 Triterpene constituents from *Z. rugosa* Lam.

Four triterpenoids, lupeol, betulin, betulinic aldehyde and betulinic acid were isolated from the $CHCl_3$ extract of the bark of *Z. rugosa* Lam [7]. β-Sitosterol and β-sitosterol glucoside were also isolated from the stem bark [8] (Figure 1.2).

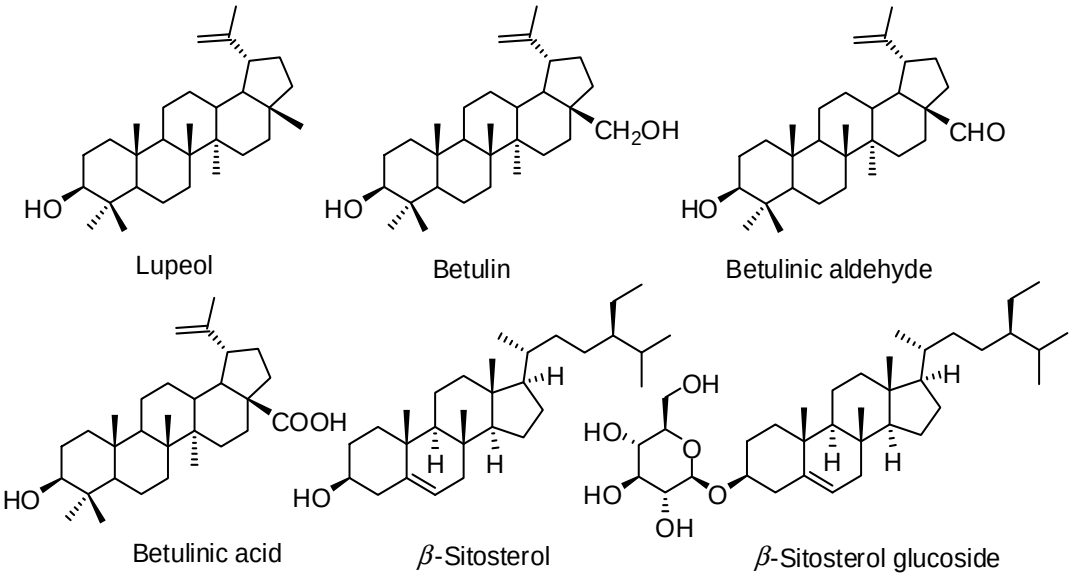


Figure 1.2 Triterpenes from *Z. rugosa* Lam.

1.1.2.2 Flavonoids constituents from *Z. rugosa* Lam.

Three flavonoids, kaempferol-4'-methylether, luteolin and luteolin-7-*O*-glucoside were isolated the first time from *Z. rugosa* Lam. Bark (Figure 1.3) [9].

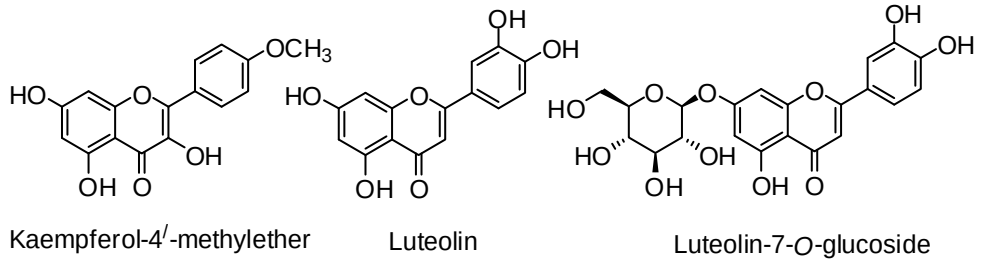


Figure 1.3 Flavoniods from *Z. rugosa* Lam.

1.1.2.3 Alkaliods constituents from *Zizyphus rugosa* Lam.

The cyclopeptide alkaloids, sativanine-C, mauritine-A, amphibine-B and nummularine-K were isolated from the bark of this plant for the frist time (Figure 1.4) [10].

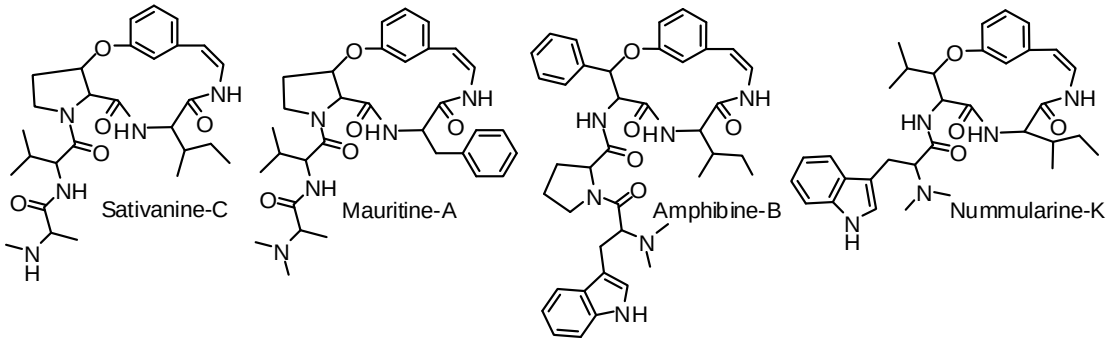


Figure 1.4 Cyclopeptide alkaloids from *Z. rugosa* Lam.

1.1.3 Botanical aspect and distribution

Ziziphus is a genus of about 40 species of spiny shrubs and small trees in the buckthorn family, Rhamnaceae, distributed in the warm-temperate and subtropical regions throughout the world. The leaves are alternate, entire, with three prominent basal veins, and 2–7 cm (0.79–2.8 in) long; some species are deciduous, others evergreen. The flowers are small, inconspicuous yellow-green. The fruit is an edible drupe, yellow-brown, red, or black, globose or oblong, 1–5

cm (0.39–2.0 in) long, often very sweet and sugary, reminiscent of a date in texture and flavour. There are about 8 species are native to Thailand, for example:

Z. calophylla Wall. (ชินชี่)

Z .cambodiana Pierre (ตะครอง)

Z. incurva Roxb. (ตาอู่แม)

Z. jujuba Mill. (พุทราจีน)

Z. mauritiana Lam. (พุทรา)

Z. oenoplia Mill. (เล็บเหยี่ยว)

Z. oenoplia Mill. var. brunoniana Tard. (หนามเล็บแมว)

Z. rugosa Lam. (มะควัด)

Z. rugosa is a species of plant in the Rhamnaceae family. It is a tree found on hills and mountains below 1,400 m altitude in China (Hainan, Yunnan), India, Laos, Burma, Sri lanka, Thailand and Vietnam**.**

The botanical aspect if this plant is shrubs or small trees, evergreen, to 9 m tall, spinose. Young branches densely ferruginous or yellow-brown tomentose; old branches red-brown, scabrous, striate, with conspicuous lenticels. Stipular spines 1(or 2), recurved, purple-red, 3-6 mm; petiole short, 5-9 mm, stout, densely yellow-brown tomentose; leaf blade broadly ovate or broadly elliptic, 8-11 × 4.5-9.5 cm, papery or subleathery, abaxially densely ferruginous or yellow-brown tomentose, adaxially at first villous, gradually glabrescent or later sparsely pubescent in vein axils, 3-5-veined from base, midvein with 2-5 pairs of secondary veins, veins prominent abaxially, impressed adaxially, base subcordate or rounded, oblique, margin serrulate, apex rounded. Inflorescences to 20 cm. Flowers green, densely pubescent, few to 10 in terminal or axillary large cymose panicles or cymose racemes; peduncles 5-12 mm. Pedicel ca. 2 mm, densely ferruginous tomentose. Sepals triangular, subequal to calyx tube, abaxially ferruginous tomentose, adaxially slightly keeled, apex acute. Petals absent. Disk orbicular, rather thick, 5-lobed, outer rim inconspicuous. Ovary globose, densely tomentose, to ca. 1/3 immersed in disk; styles deeply 2-cleft or 2-cleft to half. Drupe orange, turning black at maturity, obovoid-globose or subglobose, 9-12 mm, 8-10 mm in diam., hairy, gradually glabrescent, with persistent tube at base; fruiting

pedicel 7-10 mm, tomentose; endocarp fragile-crustaceous; stone 1-loculed, 1-seeded. Seeds red-brown, globose, 6-7 × 6-7 mm [11].

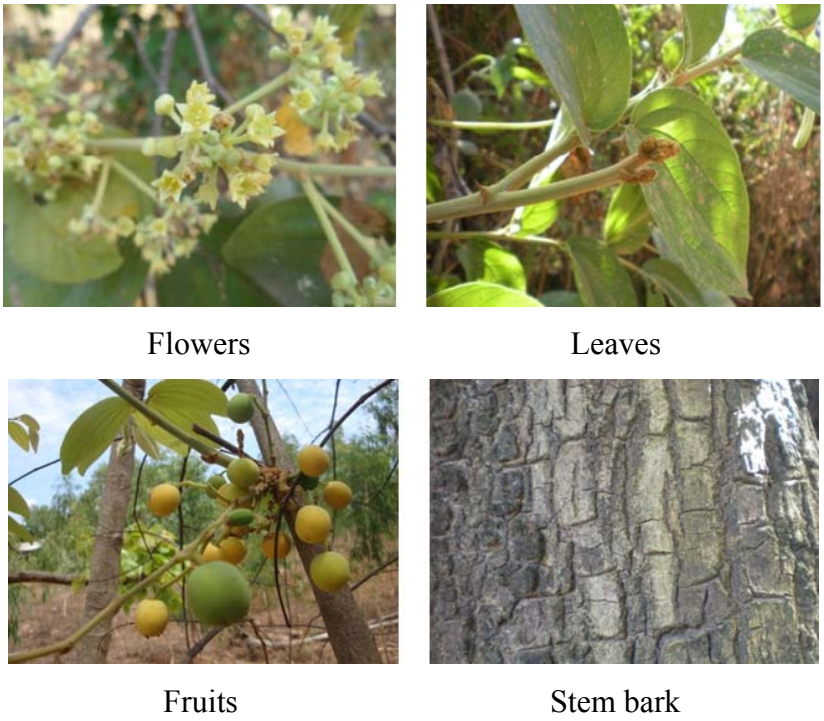


Figure 1.5 The flowers, leaves, fruits and stem bark of *Z. rugosa* Lam.

1.1.4 Biological activities

1.1.4.1 Cytotoxicity against KB and HeLa cell lines

Cytotoxicity can be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use ATP content as a marker of viability [12]. Such ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction [13]. Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and clonogenic assay. A label-free approach to follow the cytotoxic response of adherent animal cells in real-time is based on electric impedance measurements when the cells are grown on gold-film

electrodes. This technology is referred to as electric cell-substrate impedance sensing (ECIS). Label-free real-time techniques provide the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays.

From the literature review on the chemical constituents, there is no report on the cytotoxicity and chemical constituents from of Z. *rugosa* Lam. roots. Thereby, the roots of this plant were attracted for further investigation.

The objectives of this research:

The main objectives of this investigation are as follows:

1. To isolate and purify compounds from the roots of Z. *rugosa* Lam.
2. To identify the chemical structures of all isolated compounds.
3. To evaluate the cytotoxicity against HeLa and KB cell lines of the isolated compounds.

1.2 Experimental

1.2.1 Plant material

The roots of *Zizyphus rugosa* Lam. were collected from Kalasin Province of Thailand in January, 2011 and identified by Ms. Suttira Khumkratok, a botanist at the Walai Rukhavej Botanical Research Institute, Mahasarakham University, where a voucher specimen (Khumkratok no. 2-11) was deposited.

1.2.2 General experimental procedures

^{1}H, ^{13}C and 2D NMR spectra were recorded on a Varian model Mercury+ 400 spectrometer and the chemical shifts was reported in parts per million (ppm), referenced to solvent residues (δ_H 7.25, δ_C 77.0 ppm for $CDCl_3$, δ_H 2.04, δ_C 29.8, 206.5 ppm for Acetone-d_6 and δ_H 4.78, δ_C 49.0 ppm for MeOD). Adsorbents such as Sephadex LH-20 and silica gel (60 Merck cat. No. 7730, 7734 and 7749) were used for quick column chromatography, open column chromatography and centrifugal thin layer chromatography (Chromatotron). Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

1.2.3 Extraction and purification

The air-dried roots of *Z. rugosa* Lam. (4.5 kg) were successively extracted in a Soxhlet with CH_2Cl_2 and MeOH, respectively. The solvents were evaporated in vacuo to afford CH_2Cl_2 crude extract (40.5 g) and MeOH crude extract (70.5 g). The CH_2Cl_2 crude extract was subjected to vacuum liquid chromatography (VLC) over silica gel (Merck Art 7730), using successive elutions of hexane, CH_2Cl_2 and MeOH with increasing polarity to afford three fractions (F1-F3).

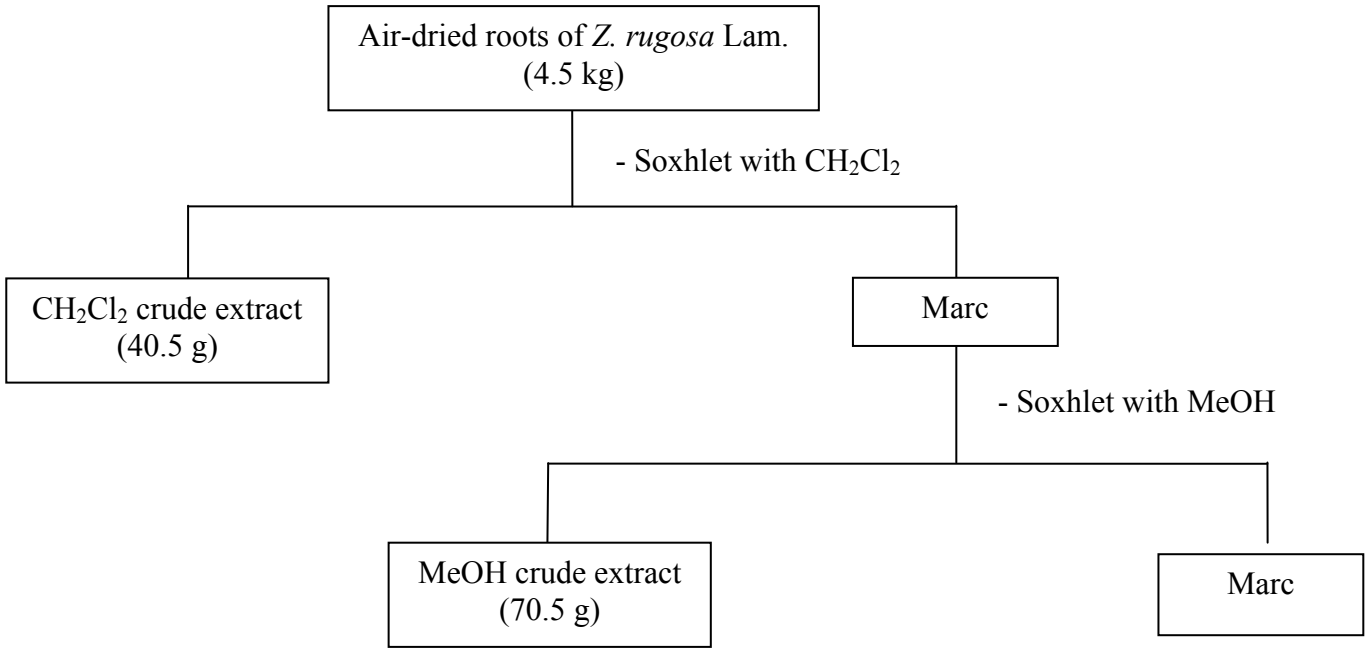
Fraction F1 (7.2 g) was chromatographed on a silica gel column eluted with CH_2Cl_2 and hexane gradient system to give five subfractions (F1.1-F1.5). Subfraction F1.3 was purified by recrystallization (MeOH : CH_2Cl_2, 2:8) to afford lupeol (**1.1**, 1.5 g) and betulinic aldehyde (**1.3** ,0.3 g). Fraction F2 (18.4 g) was subjected to silica gel column, using a gradient system between CH_2Cl_2-hexane to afford four subfractions (F2.1-F2.4). Subfraction F2.2 was further separated by silica gel column eluted with hexane, CH_2Cl_2 and MeOH gradient system and recrystalized (MeOH:CH_2Cl_2, 2:8) to

give betulin (**1.2**, 1.2 g). F2.3 was separated by Sephadex LH-20 to give two subfractions (F2.3.1 and F2.3.2). Betulinic acid (**1.4**, 1.1 g) and euscaphic acid (**1.6**, 0.5 g) were rechromatographed from subfraction F2.3.1 by Cromatotron (Hexane:EtOAc, 1:1). F2.3.1 was recrystallized on 1:5 of MeOH:CH_2Cl_2 to afford 3 g of a mixure of *β*-sitosterol (**1.8**) and stigmasterol (**1.9**). F2.4 was applied to Chromatotron using hexane and EtOAc as a gradient system yielding zizyberenalic acid (**1.7**, 0.7 g) and scopolitin (**1.10**, 3 mg). Finally, fraction F3 was chromatographed on Sephadex LH-20 using gradient of CH_2Cl_2 and MeOH to afford alphitolic acid (**1.5**, 0.6 g) (Figure 1.6).

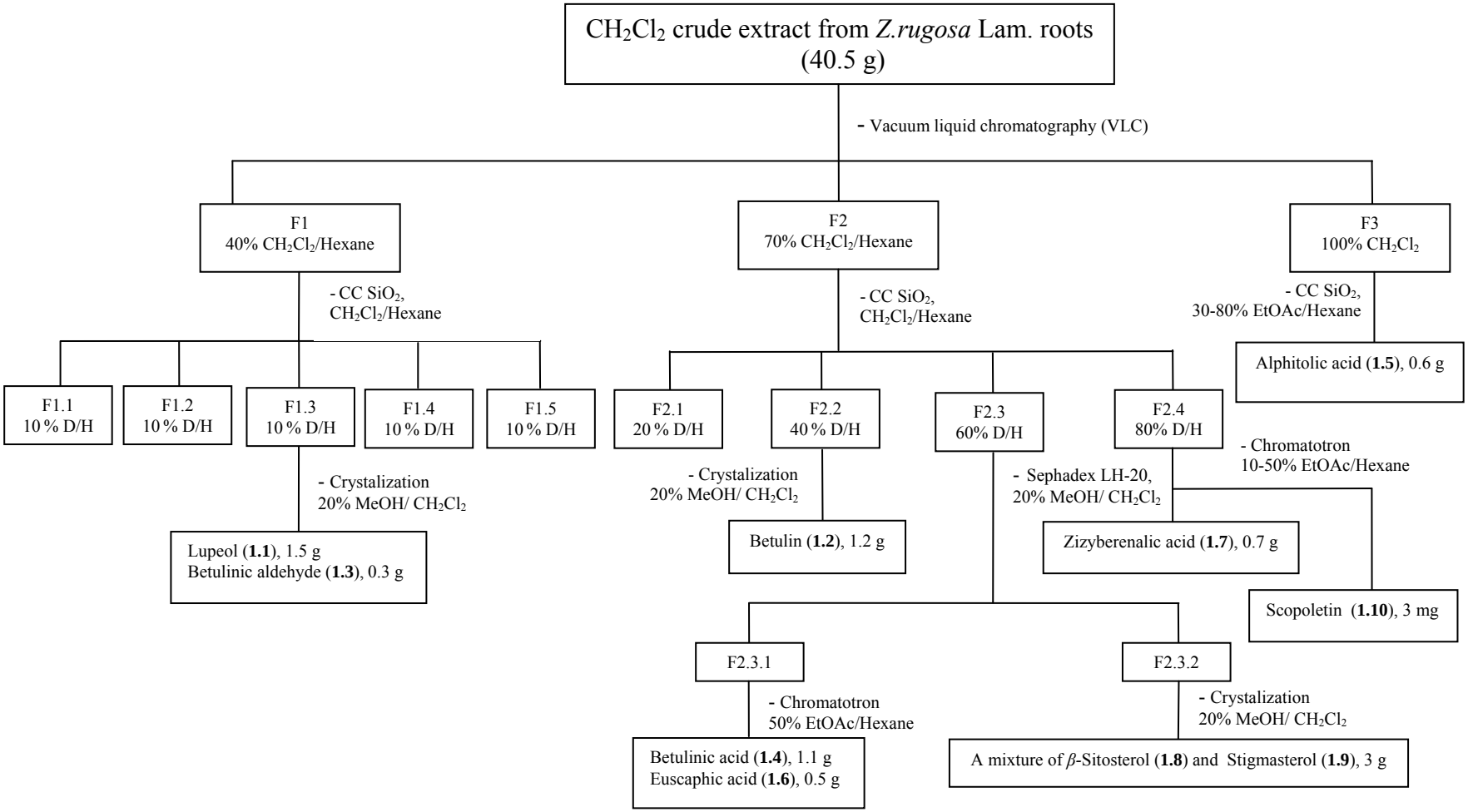
The MeOH crude extract was dissolved in water and the aqueous layer of *Z. rugosa* Lam. was subjected of Dianion HP-20 column and successively eluted with water, MeOH and acetone. The MeOH soluble extract from dissolved in water (50.0 g) was subjected to silica gel column and was eluted with EtOAc and MeOH gradient system to give three fractions (M1-M3). Kaemferol (**1.11**, 60 mg) was separated by Chromatotron eluted with 1:1 of hexane:EtOAc from fraction M1. Fractions M2 and M3 were purified by similar condition as M1 to give afzelin (**1.12**, 1.5 mg), quercitrin (**1.13**, 70 mg) and (+)-catechin (**1.14**, 80 mg), respectively (Figure 1.7).

The identification of all of isolated compounds was determined by mean of various michoscopic methods including MS, 1D and 2D NMR literature as well as comparision with the literature data.

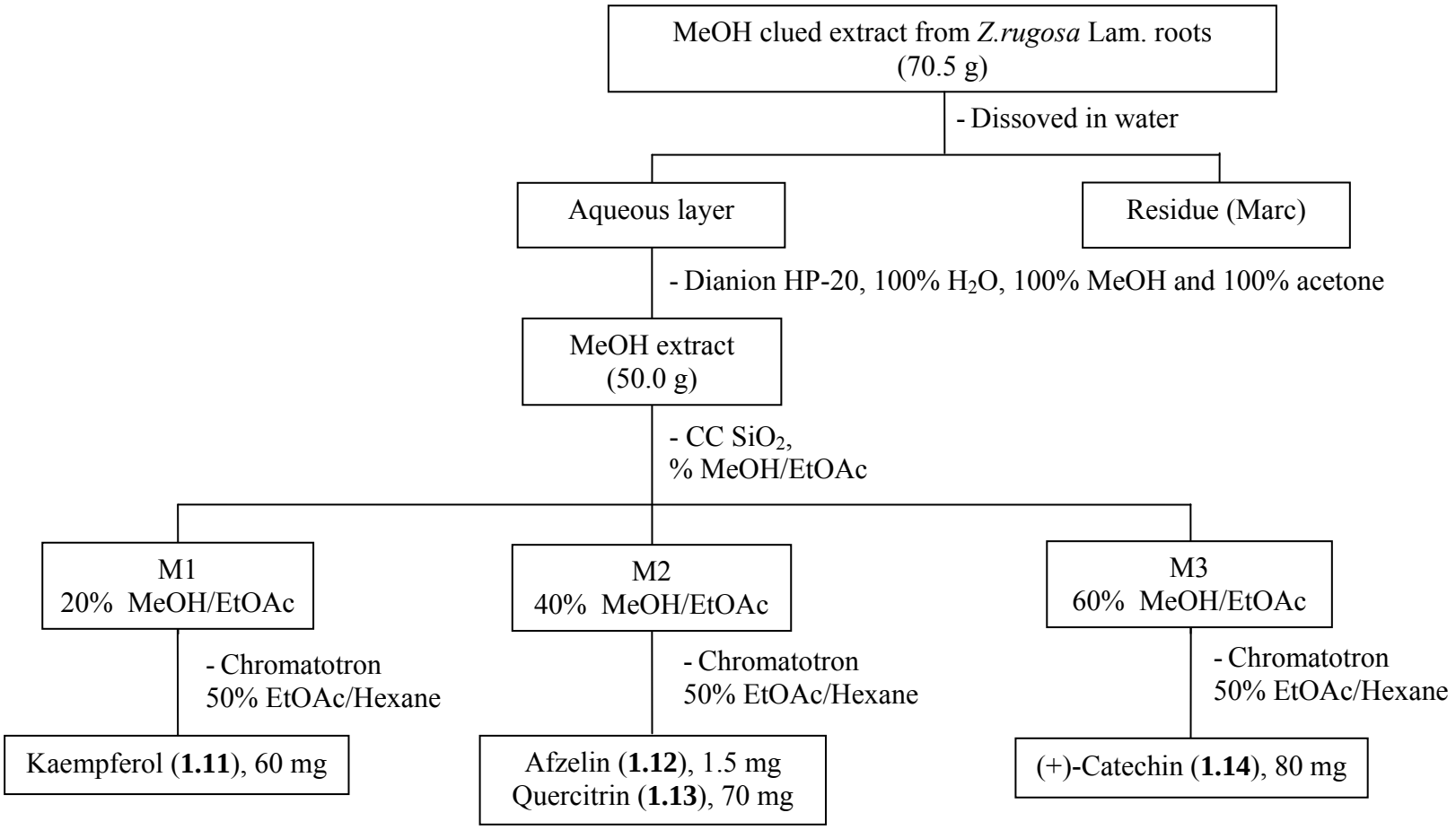
The isolation and purification of all isolated compounds from the CH_2Cl_2 and MeOH extract of the roots of *Z. rugosa* Lam. were briefly summarized in scheme 1.1-1.3.



Scheme 1.1 Extraction procedure of *Z. rugosa* Lam. roots.



Scheme 1.2 Isolation procedure of the CH_2Cl_2 crude extract from *Z.rugosa* roots.



Scheme 1.3 Isolation procedure of the MeOH crude extract from *Z.rugosa* roots.

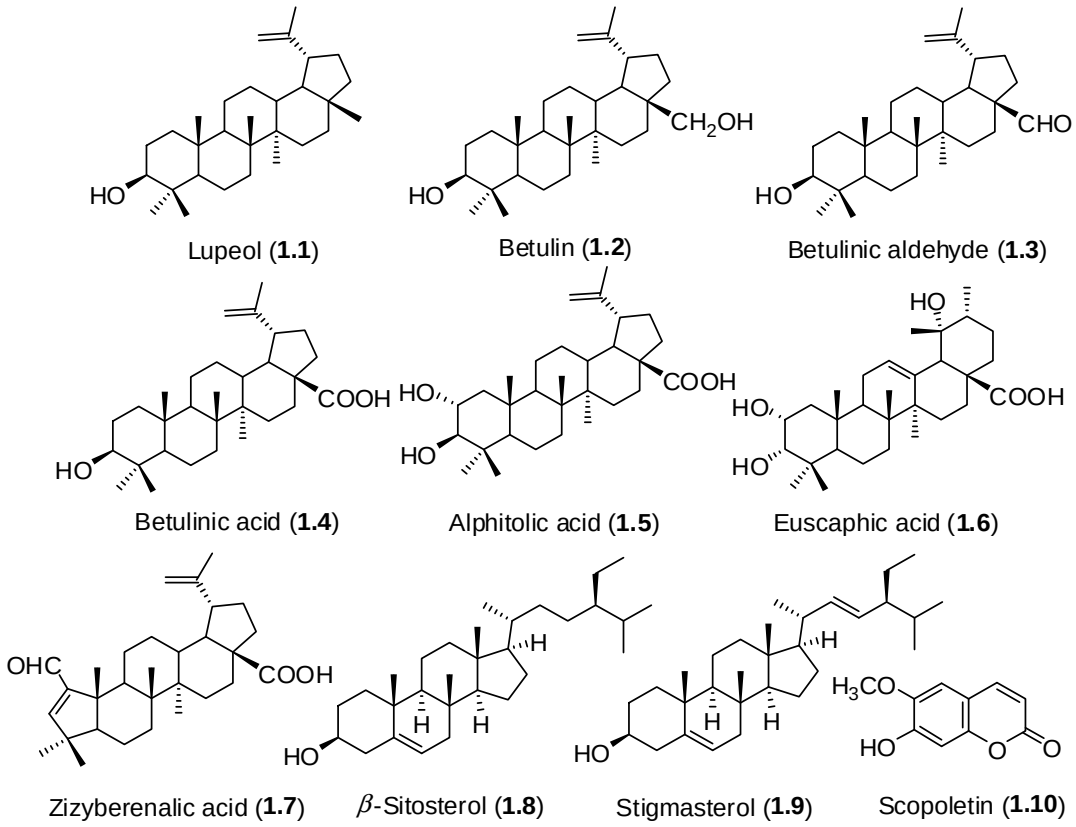


Figure 1.6 Isolated compounds from the CH_2Cl_2 crude extract of *Z. rugosa* Lam. roots.

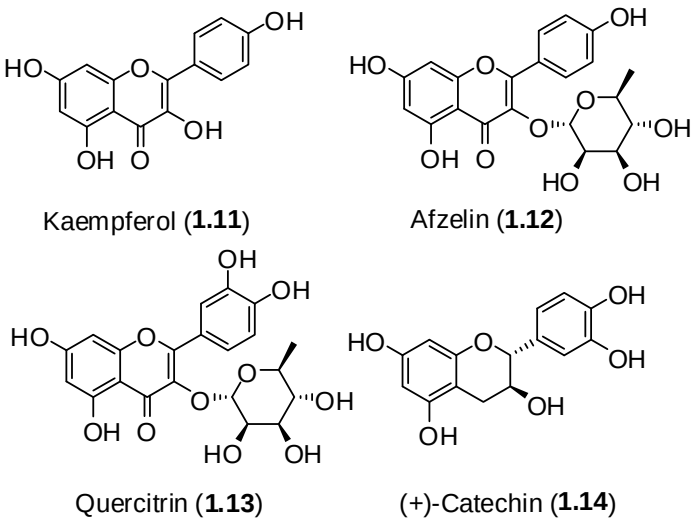


Figure 1.7 Isolated compounds from the MeOH crude extract of *Z. rugosa* Lam. Roots

1.2.4 Bioassay procedure

1.2.4.1 The cytotoxicity against HeLa and KB cell lines by MTT colorimetric assay

All tested compounds (1 mg each) were subjected to cytotoxic evaluation against KB (human epidermoid carcinoma) and HeLa (human cervical carcinoma) cell lines employing the MTT colorimetric assay. Adriamycin was used as standard antibiotic antitumor agent which exhibits activity against KB and HeLa cell lines according to the method of Kongkathip *et al.* [14]. This assay was kindly performed by Natural Products Research Section, Research Division, National Cancer Institute, Thailand.

1.3 Results and Discussion

1.3.1 Properties and structural elucidation of isolated compounds

1.3.1.1 Alphitolic acid (1.5)

Alphitolic acid (**1.5**) was obtained as a colorless needle. The chemical formula was established as $C_{30}H_{48}O_4$ from ^{1}H and ^{13}C NMR. The ^{1}H NMR spectrum of **1.5** showed in Table 1.1 displayed resonances for six singlet methyl groups at δ_H 0.65 (3H, s, H-23), 0.78 (3H, s, H-25), 0.82 (3H, s, H-26), 0.86 (3H, s, H-24), 0.89 (3H, s, H-27) and 1.57 (3H, d, H-29), and two *exo*-methylene protons at δ_H 4.42 (1H, s, H-30α) and 4.50 (1H, s, H-30β), two oxygenated methine protons at δ_H 2.77 (1H, d, J = 9.0 Hz, H-3) and 3.46 (1H, m, H-2). In the ^{13}C NMR spectrum, 30 carbon signals were observed, including six methyls (δ_C 15.0, 16.5, 17.1, 17.8, 19.4, and 29.0), two oxygenated methines (δ_C 69.1 and 83.9), two *exo*-methylene carbons (δ 151.6 and 110.0 and a carboxylic group (δ_C 177.5). The HMBC spectrum showed the correlations (Figure 1.9) closely resemble those of alphitolic acid Thus, the structure of **1.5** was determined to be alphitolic acid (Figure 1.8). To the best of my knowledge, this compound was isolated for the first time from this plant.

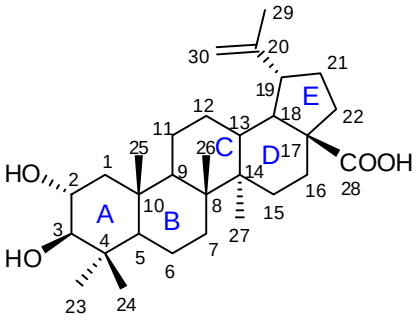


Figure 1.8 Structure of alphitolic acid (**1.5**).

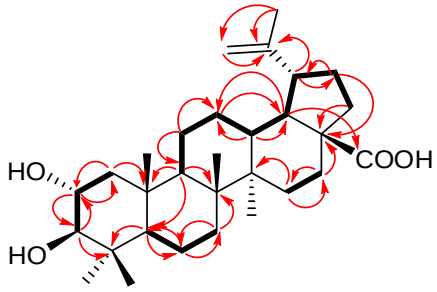


Figure 1.9 Selected HMBC (arrow curves) and COSY (bold lines) correlations of (**1.5**).

Table 1.1 ^{1}H, ^{13}C NMR and HMBC data of **1.5** in acetone-d_6 (400 MHz for ^{1}H, 100 MHz for ^{13}C).

Position	δ_H (mult, J in Hz)	δ_C	HMBC
1	1.85 (2H, m)	47.9	C-2, C-3, C-5, C-10, C-25
2	3.46 (1H, m)	69.1	C-3
3	2.77 (1H, d, J = 9.0 Hz)	83.9	C-1, C-2, C-4, C-23, C-24
4	-	39.8	-
5	-	56.3	C-4, C-6, C-9, C-25
6	1.38 (2H, m)	19.0	C-5, C-7, C-8
7	1.23 (2H, m),	31.3	C-5,C-6, C-8, C-9, C-10, C-27
8	-	41.6	-
9	-	51.3	C-1, C-5, C-8, C-10
10	-	37.5	-
11	1.36 (2H, m)	21.8	C-8, C-9, C-10, C-12, C-13, C-14
12	1.60 (2H, m)	26.3	C-18
13	2.22 (1H, m)	39.2	C-12, C-14, C-18, C-27
14	-	43.2	-
15	1.16 (2H, m)	30.2	C-7, C-8, C-12, C-13, C-16, C-22
16	2.13 (2H, m)	32.8	C-14, C-15, C-17, C-18
17	-	56.7	-
18	1.50 (1H, m)	49.9	C-12, C-16, C-28
19	2.92 (1H, m)	47.8	C-13, C-18, C-20, C-21, C-29, C-30
20	-	151.6	-
21	1.06 (2H, m)	30.4	C-19, C-22, C-27
22	1.25 (2H, m)	35.1	C-16, C-17, C-19, C-20
23	0.65 (3H, s)	17.1	C-3, C-4, C-5, C-24
24	0.86 (3H, s)	29.0	C-2, C-3, C-4, C-5, C-23
25	0.78 (3H, s)	17.8	C-9
26	0.82 (3H, s)	16.5	C-8, C-9, C-14
27	0.89 (3H, s)	15.0	C-8, C-13, C-14, C-15
28	-	177.5	-
29	1.57 (3H, s)	19.4	C-19, C-20, C-30
30α	4.42 (1H, s)	110.0	C-18, C-29
30β	4.50 (1H, s)	110.0	C-18, C-29

1.3.1.2 Euscaphic acid (1.6)

Euscaphic acid (**1.6**) was obtained as a colorless needle. Interpretation of the ^{1}H and ^{13}C NMR spectral data of **1.6** with the use of ^{1}H-^{1}H COSY, HMQC and HMBC spectra as well as compare with the literature. The ^{1}H NMR spectrum of **1.6** showed signals for typical seven tertiary methyl groups δ_H 0.67, 0.74, 0.84, 0.87, 0.88, 1.11, 1.25) a signal of H-3 ax at δ_H 3.20 (1H, d, J = 11.5 Hz), signal of H-2 ax at δ_H 3.82 (1H, m), and a olefinic proton at δ 5.18 (1H, t, J = 3.5 Hz). The ^{13}C NMR spectrum showed 30 signals that could be correlated to the corresponding proton chemical shifts from the HSQC experiment. In the 2D NMR data (Table 1.2), which showed the HMBC correlations between H-3 and C-2, C-4, C-23, C-24; H-29 and C-18, C-19, C-20; H-30 and C-19, C-20, C-21 (Figure 1.11). Based on the 1D and 2D NMR analysis as well as comparison with previous literatures, it strongly suggested that **1.6** has the characteristic as same as euscaphic acid (Figure 1.10). To the best of my knowledge, this compound was isolated for the first time from this plant.

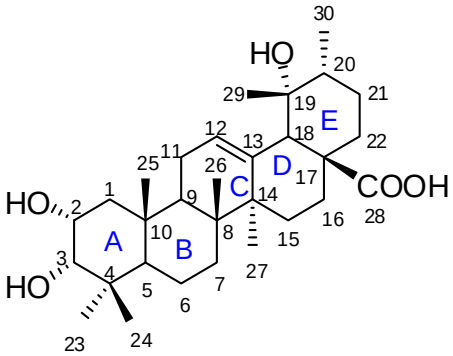


Figure 1.10 Structure of euscaphic acid (**1.6**).

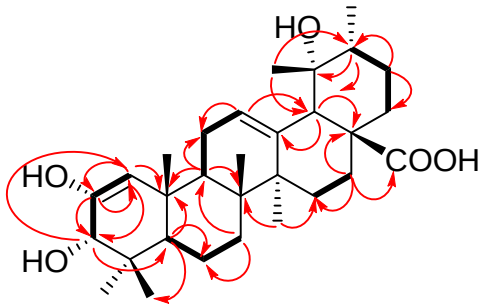


Figure 1.11 Selected HMBC (arrow curves) and COSY (bold lines) correlations of **1.6**.

Table 1.2 ^{1}H, ^{13}C NMR and HMBC data of **1.6** in acetone-d_6 (400 MHz for ^{1}H, 100 MHz for ^{13}C).

Position	δ_H (mult, J in Hz)	δ_C	HMBC
1	1.48 (2H, m)	42.7	C-2, C-3, C-5, C-10, C-25
2	3.82 (1H, m)	66.3	C-1, C-3
3	3.20 (1H, d, J = 11.5 Hz)	79.4	C-1, C-2, C-5
4	-	38.4	-
5	1.21 (1H, s)	48.9	C-6, C-9, C-10, C-23, C-24, C-25
6	1.34 (2H, m)	18.9	C-5, C-7
7	1.21 (2H, m)	33.8	C-6, C-9
8	-	40.8	-
9	1.69 (1H, m)	47.9	C-8, C-10, C-11, C-26
10	-	38.8	-
11	1.93 (2H, m)	24.7	C-12
12	5.18 (1H, t, J = 3.5 Hz)	128.8	C-9, C-11, C-14, C-18
13	-	139.6	-
14	-	42.3	-
15	1.71 (2H, m)	29.1	C-16
16	2.53 (2H, m)	26.4	C-15, C-17, C-28
17	-	48.3	-
18	2.44 (1H, s)	54.5	C-12, C-13, C-14, C-16, C-17, C-19
19	-	73.3	-
20	1.28 (1H, m)	42.6	-
21	1.14 (2H, m)	27.0	C-18, C-19, C-20, C-22
22	1.62 (2H, m)	38.9	C-21
23	0.74 (3H, s)	22.3	C-3, C-4, C-5, C-24
24	0.88 (3H, s)	29.1	C-3, C-4, C-5, C-23
25	0.87 (3H, s)	16.7	-
26	0.67 (3H, s)	17.4	C-7, C-8, C-9, C-14
27	1.25 (3H, s)	24.3	C-8, C-14, C-15
28	-	179.1	-
29	1.11 (3H, s)	27.3	C-18, C-19, C-20
30	0.84 (3H, s)	16.5	C-19, C-20

1.3.1.2 Zizyberenalic acid (1.7)

Zizyberenalic acid (**1.7**) was afforded as a colorless powder. It had a molecular formula $C_{30}H_{44}0_3$, which was deduced from HRESI mass spectrum. The ^{1}H NMR spectrum (Table 1.3) displayed proton signals in the downfield region for δ_H 9.86 s, H-2 and 6.49 s, H-3 that correspond to ^{13}C NMR at δ_C 190.3 (C-2), 156.3 (C- 1) and 162.2 (C-3), respectively. These data suggested **1.7** to be a 1,3-didehydro derivative of ceanothic acid, which was also confirmed by the HMBC correlation of C-19 with methyl protons (Figure 1.13). The HMBC experiments showed the correlations of 9.86 (1H, s) to C-1, C-10 and 6.49 (1H, s) to C-1, C-2, C-4, C-5 and C-10 was also proved the partial structure in ring A. The rest of the structure is the same as that of ceanothic acid. These evidences were compared with ^{1}H, ^{13}C and 2D NMR data from the literature data, which revealed that **1.7** is zizyberenalic acid (Figure 1.12). To the best of my knowledge, this compound was isolated for the first time from this plant.

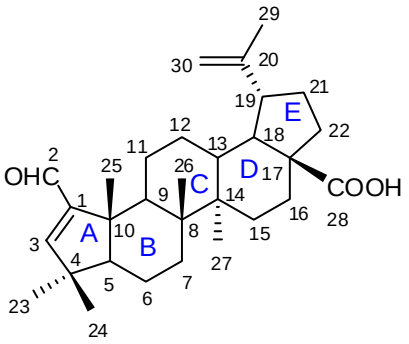


Figure 1.12 Structure of zizyberenalic acid (**1.7**).

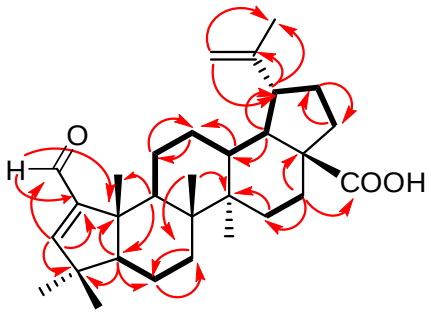


Figure 1.13 Selected HMBC (arrow curves) and COSY (bold lines) correlations of **1.7**.

Table 1.3 ^{1}H, ^{13}C NMR and HMBC data of **1.7** in $CDCl_3$ (400 MHz for ^{1}H, 100 MHz for ^{13}C).

Position	δ_H (mult, *J* in Hz)	δ_C	HMBC
1	-	156.3	-
2	9.86 (1H, s)	190.3	C-1, C-10
3	6.49 (1H, s)	162.2	C-1, C-2, C-4, C-5, C-10
4	-	42.7	-
5	1.40 (1H, m)	62.0	C-4, C-6, C-7, C-10, C-23
6	-	15.8	C-7
7	-	34.1	C-6
8	-	41.9	-
9	-	46.5	C-5, C-25
10	-	51.1	-
11	-	23.1	C-12
12	-	24.1	C-11
13	2.70 (1H, dt, *J* = 3.5, 12.5 Hz)	37.1	C-12, C-18
14	-	41.5	-
15	-	28.8	C-14, C-16, C-27
16	2.61 (2H, brd, *J* = 12.6 Hz)	31.3	C-15, C-17, C-28
17	-	55.0	-
18	1.71 (1H, m)	48.4	C-13, C-17, C-19, C-20
19	3.47 (1H, dt, *J* = 11.0, 3.9 Hz)	45.9	C-18, C-29
20	-	149.0	-
21	-	29.5	C-22
22	-	36.0	C-21, C-17
23	1.09 (3H, s)	27.1	C-4, C-5, C-24
24	0.88 (3H, s)	19.4	C-4, C-5, C-23
25	1.11 (3H, s)	18.0	C-1, C-5, C-9, C-10
26	1.07 (1H, s)	16.6	C-7, C-8, C-9, C-14
27	0.99 (1H, s)	13.7	C-8, C-13, C-14, C-15
28	-	178.4	-
29	1.77 (3H, s)	18.1	C-19, C-20, C-30
30	4.89 (2H, brs)	108.9	C-19, C-29

1.3.1.4 Betulin (1.2)

Betulin (**1.2**) was isolated from this plant and possessed good antioxidant activity. The identification of **1.2** (Figure 1.14) was determined by means of spectroscopic methods (MS, ^{1}H, ^{13}C NMR and 2D NMR) as well as comparison with literature data. The exact molecular structure of **1.2** was also confirmed by X-ray crystallography (Figure 1.15).

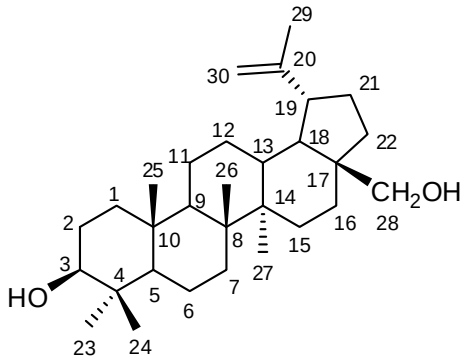


Figure 1.14 Structure of botulin (**1.2**).

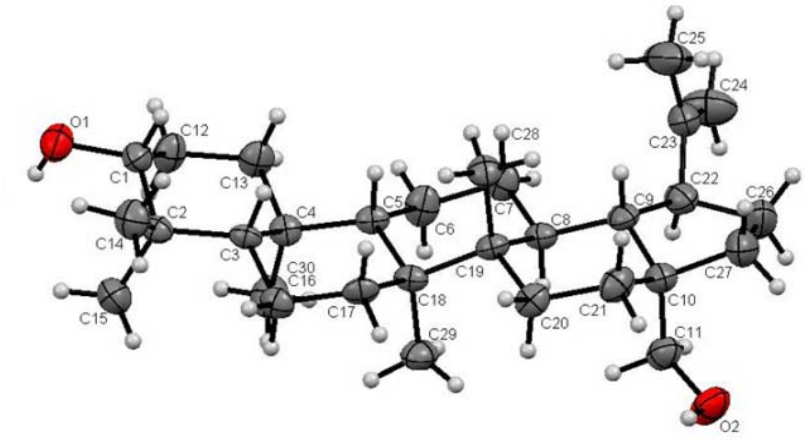
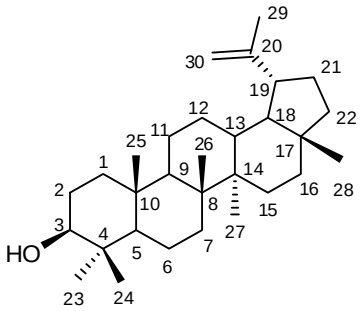
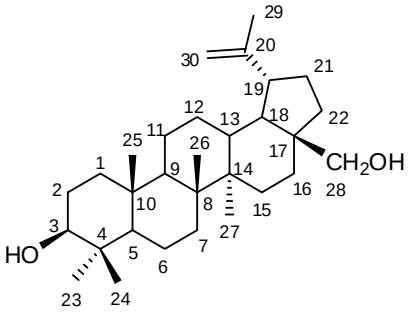


Figure 1.15 ORTEP view of x-ray molecular structure of betulin (**1.2**).

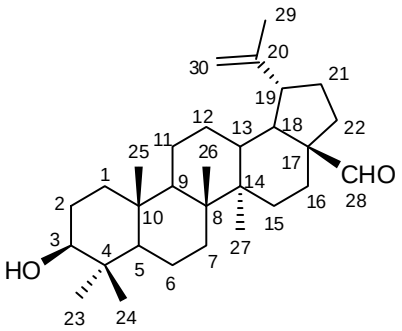


Lupeol (1.1): colorless needles; ^{1}H NMR ($CDCl_3$): δ_H 0.66 (1H, d, *J* = 9.1 Hz, H-5), 0.73 (3H, s, H-24), 0.76 (3H, s, H-28), 0.80 (3H, s, H-25), 0.92 (3H, s, H-27), 0.94 (3H, s, H-23), 1.00 (3H, s, H-26), 1.65 (3H, s, H-29), 1.82-1.96 (2H, m, H-21), 2.35 (1H, dt, *J* = 10.9, 5.5 Hz, H-19), 3.16 (1H, dd, *J* = 10.8, 5.1 Hz, H-3), 4.55 (1H, brs, H-30), 4.65 (1H, brs, H-30); ^{13}C NMR ($CDCl_3$): δ_C 14.5 (C-27), 15.3 (C-24), 15.9 (C-25), 16.1 (C-26), 18.0 (C-28), 18.3 (C-6), 19.3 (C-29), 20.9 (C-11), 25.1 (C-12), 27.4 (C-2, C-15), 28.0 (C-23), 29.7 (C-21), 34.3 (C-7), 35.6 (C-16), 37.1 (C-10), 38.0 (C-13), 38.7 (C-1), 38.8 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 43.0 (C-17), 48.0 (C-18), 48.3 (C-19), 50.4 (C-9), 55.3 (C-5), 79.0 (C-3), 109.3 (C-30), 150.9 (C-20) [15].

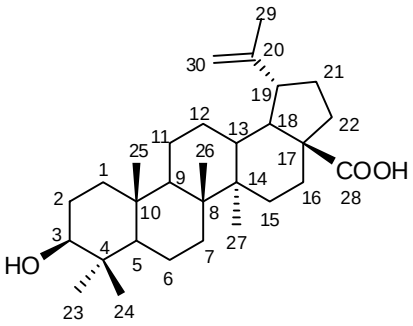


Betulin (1.2): colorless crystals; ^{1}H NMR ($CDCl_3$): δ_H 0.65 (1H, d, *J* = 9.4 Hz, H-5), 0.73 (3H, s, H-24), 0.79 (3H, s, H-25), 0.94 (3H, s, H-27), 0.95 (3H, s, H-23), 0.99 (3H, s, H-26), 1.65 (3H, s, H-29), 2.35 (1H, dt, *J* = 10.5, 6.2 Hz, H-19), 3.16 (1H, dd, *J* = 10.8, 4.9 Hz, H-3), 3.30 (1H, d, *J* = 10.8, H-28), 3.77 (1H, d, *J* = 10.8 Hz, H-28), 4.55 (1H, brs, H-30), 4.65 (1H, brs, H-30); ^{13}C NMR ($CDCl_3$): δ_C 14.7 (C-27), 15.3 (C-24), 15.9 (C-26), 16.1 (C-25), 18.3 (C-6), 19.0 (C-29), 20.8 (C-11), 25.2 (C-12), 27.0 (C-15), 27.4 (C-2), 27.9 (C-23), 29.2 (C-16), 29.7 (C-21), 33.9 (C-22), 34.2 (C-7), 37.1 (C-10), 37.3 (C-13), 38.7 (C-1), 38.8 (C-4), 40.9 (C-8), 42.7 (C-14), 50.8 (C-

17), 48.7 (C-18), 47.8 (C-19), 50.4 (C-9), 55.3 (C-5), 60.5 (C-28), 79.0 (C-3), 109.6 (C-30), 150.4 (C-20) [15].

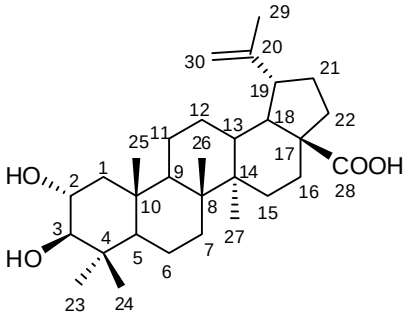


Betulinic aldehyde (1.3): colorless needles; ^{1}H NMR ($CDCl_3$): δ_H 0.64 (1H, d, *J* = 9.3 Hz, H-5), 0.74 (3H, s, H-24), 0.78 (3H, s, H-25), 0.92 (3H, s, H-27), 0.93 (3H, s, H-23), 0.98 (3H, s, H-26), 1.64 (3H, s, H-29), 2.86 (1H, dt, *J* = 10.4, 6.1 Hz, H-19), 3.15 (1H, dd, *J* = 10.7, 4.8 Hz, H-3), 4.54 (1H, brs, H-30), 4.63 (1H, brs, H-30), 9.65 (1H, s, H-28); ^{13}C NMR ($CDCl_3$): δ_C 14.6 (C-27), 15.4 (C-24), 16.2 (C-25), 16.3 (C-26), 18.3 (C-6), 19.0 (C-29), 20.9 (C-11), 25.5 (C-12), 27.9 (C-23), 27.6 (C-2), 29.8 (C-21), 30.5 (C-15), 32.5 (C-16), 34.4 (C-7), 37.2 (C-22), 37.7 (C-10), 38.1 (C-13), 39.1 (C-1), 39.2 (C-4), 40.7 (C-8), 42.4 (C-14), 47.0 (C-18), 49.3 (C-19), 50.6 (C-9), 55.4 (C-5), 56.2 (C-17), 78.9 (C-3), 110.0 (C-30), 149.7 (C-20), 206.6 (C-28) [16].

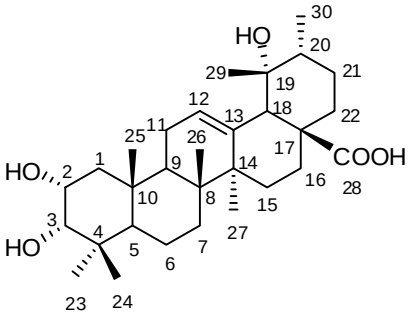


Betulinic acid (1.4): colorless powder ; ^{1}H NMR ($CDCl_3$): δ_H 0.65 (1H, d, *J* = 8.2 Hz, H-5), 0.72 (3H, s, H-24), 0.79 (3H, s, H-25), 0.90 (3H, s, H-26), 0.94 (3H, s, H-23), 0.95 (3H, s, H-27), 1.66 (3H, s, H-29), 2.98 (1H, m, H-19), 3.16 (1H, dd, *J* = 10.9, 4.9 Hz, H-3), 4.58 (1H, brs, H-30), 4.71 (1H, brs, H-30); ^{13}C NMR ($CDCl_3$): δ_C 14.7 (C-27), 15.4 (C-24), 16.0 (C-25), 16.1 (C-26), 18.3 (C-6), 19.3 (C-29), 20.8 (C-11), 25.5 (C-12), 27.4 (C-23), 27.9 (C-2), 29.7 (C-21), 30.5 (C-15), 32.1 (C-16), 34.3 (C-7), 37.0 (C-22), 37.2 (C-10), 38.4 (C-13), 38.7 (C-1), 38.8 (C-4), 40.7 (C-8), 42.4 (C-14),

46.8 (C-18), 49.3 (C-19), 50.5 (C-9), 55.3 (C-5), 56.2 (C-17), 79.0 (C-3), 109.6 (C-30), 150.3 (C-20), 179.4 (C-28) [16].

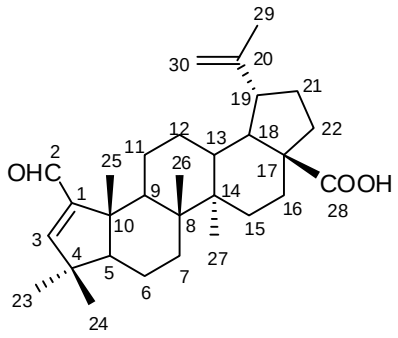


Alphitolic acid (1.5): colorless needles; ^{1}H NMR (Acetone-d_6): δ_H 0.65 (3H, s, H-23), 0.78 (3H, s, H-25) 0.82 (3H, s, H-26), 0.86 (3H, s, H-24), 0.89 (3H, s, H-27), 1.06 (2H, m, H-21), 1.16 (2H, t, H-15), 1.23 (2H, m, H-7), 1.25 (2H, m, H-22), 1.36 (2H, m, H-11), 1.38 (2H, m, H-6), 1.50 (1H, m, H-18), 1.57 (3H, s, H-29), 1.60 (2H, m, H-12), 1.85 (2H, m, H-1), 2.13 (2H, dd, H-16), 2.77 (1H, m, H-3), 3.46 (1H, m, H-2), 4.42 (1H, brs, H-30), 4.50 (1H, brs, H-30); ^{13}C NMR (Acetone-d_6): δ_C 15.0 (C-27), 16.5 (C-26), 17.1 (C-23), 17.8 (C-25), 19.0 (C-6), 19.4 (C-29), 21.8 (C-11), 26.3 (C-12), 29.0 (C-24), 30.2 (C-15), 30.4 (C-21), 31.3 (C-7), 32.8 (C-16), 35.1 (C-22), 37.5 (C-10), 39.1 (C-13), 39.8 (C-4), 41.6 (C-8), 43.2 (C-14), 47.8 (C-19), 47.9 (C-1), 49.9 (C-18), 51.3 (C-9), 56.3 (C-5), 56.7 (C-17), 69.1 (C-2), 83.9 (C-3), 110.0 (C-30), 151.6 (C-20), 177.5 (C-28) [17].

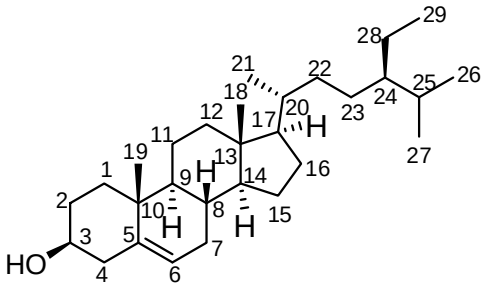


Euscaphic acid (1.6): colorless needles; ^{1}H NMR (Acetone-d_6): δ_H 0.67 (1H, s, H-26), 0.74 (3H, s, H-23), 0.84 (3H, d, H-30), 0.87 (3H, s, H-25), 0.88 (3H, s, H-24), 1.11 (3H, s, H-29), 1.21 (1H, s, H-5), 1.25 (1H, s, H-27), 3.20 (1H, d, J = 10.6, H-3), 3.82 (1H, m, H-2), 5.18 (1H, t, J=3.5 Hz); ^{13}C NMR (Acetone-d_6): δ_C 16.5 (C-30), 16.7 (C-25), 17.4 (C-26), 18.9 (C-6), 22.3 (C-24), 24.3 (C-27), 24.7 (C-11), 26.4 (C-16), 27.0 (C-21), 27.3 (C-29), 29.1 (C-15), 29.2 (C-23), 33.8 (C-7), 38.4 (C-4), 38.8

(C-10), 38.9 (C-22), 40.8 (C-8), 42.3 (C-14), 42.6 (C-20), 42.7 (C-1), 47.9 (C-9), 48.3 (C-17), 48.9 (C-5), 54.5 (C-18), 66.3 (C-2), 73.3 (C-19), 79.4 (C-3), 128.8 (C-12), 139.6 (C-13), 179.1 (C-28) [18].

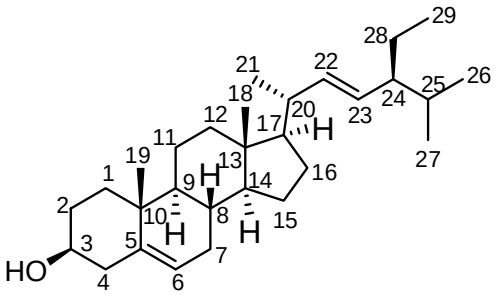


Zizyberenalic acid (1.7): colorless powder ; ^{1}H NMR ($CDCl_3$): δ_H 0.88 (3H, s, H-24), 0.99 (1H, s, H-27), 1.07 (1H, s, H-26), 1.09 (3H, s, H-23), 1.11 (3H, s, H-25), 1.71 (1H, m, H-18), 1.77 (3H, s, H-29), 2.61 (2H, brd, *J* = 12.6 Hz, H-16), 2.70 (1H, dt, *J* = 3.5, 12.5 Hz, H-13), 3.47 (1H, dt, *J* = 11.0, 3.9 Hz, H-19), 4.89 (2H, brs, H-30), 9.86 (1H, s, H-2); ^{13}C NMR ($CDCl_3$): δ_C 13.7 (C-27), 15.8 (C-6), 16.6 (C-26), 18.0 (C-25), 18.1 (C-29), 18.2 (C-29), 19.4 (C-24), 23.1 (C-11), 24.1 (C-12), 27.1 (C-23), 28.8 (C-15), 29.5 (C-21), 31.3 (C-16), 34.1 (C-7), 36.0 (C-22), 37.1 (C-13), 41.5 (C-14), 41.9 (C-8), 42.7 (C-4), 45.9 (C-19), 46.5 (C-9), 48.4 (C-18), 51.1 (C-10), 55.0 (C-17), 62.0 (C-5), 108.9 (C-30), 109.3 (C-2), 149.0 (C-20), 156.3 (C-1), 162.2 (C-3), 178.4 (C-28) [19].

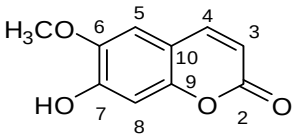


***β*-Sitosterol (1.8)**: colorless needles, ^{1}H NMR ($CDCl_3$) δ_H 0.72 (3H, s, CH_3-18), 0.85 (3H, d, *J* = 6.7 Hz, CH_3-27), 0.87 (3H, d, *J* = 6.7 Hz, CH_3-26), 0.89 (3H, t, *J* = 7.4 Hz, CH_3-29), 0.96 (3H, *d*, *J* = 6.5 Hz, CH_3-21), 1.05 (3H, s, CH_3-19), 5.39 (1H, m, H-6); ^{13}C NMR ($CDCl_3$): δ_C 12.3(C-29), 12.4 (C-18), 19.2 (C-21), 19.5 (C-27), 19.8 (C-19), 20.2 (C-26), 21.5 (C-11), 23.3 (C-28), 24.7 (C-15), 26.5 (C-23), 28.7(C-16), 29.6 (C-25), 32.1 (C-7), 32.3 (C-2,8), 34.4 (C-22), 36.6 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2

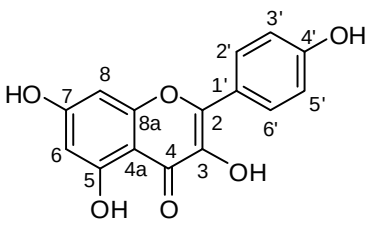
(C-12), 42.8 (C-4,13), 46.2 (C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 72.2 (C-3), 122.1 (C-6), 141.2 (C-5) [20].



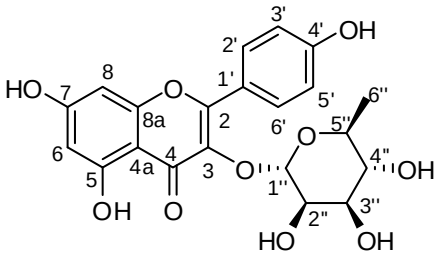
Stigmasterol (1.9): colorless needles, ^{1}H NMR ($CDCl_3$) δ_H 0.72 (3H, *s*, CH_3-18), 0.85 (3H, *d*, *J* = 6.7 Hz, H-27), 0.87 (3H, d, *J* = 6.7 Hz, H-26), 0.89 (3H, *t*, *J* = 7.4 Hz, CH_3-29), 0.96 (3H, *d*, *J* = 6.5 Hz, CH_3-21), 1.05 (3H, s, CH_3-19), 3.56 (1H, m, H-3),5.01 (1H, m, H-22), 5.15 (1H, m, H-23) 5.39 (1H, m, H-6); ^{13}C NMR ($CDCl_3$): δ_C 12.3 (C-29), 12.4 (C-18), 19.2 (C-21), 19.5 (C-27), 19.8 (C-19), 20.2 (C-26), 21.4 (C-23) 21.5 (C-11), 23.3 (C-28), 24.7 (C-15), 28.7(C-16), 29.6 (C-25), 32.1 (C-7), 32.3 (C-2,8), 36.6 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2 (C-12), 40.7 (C-22), 42.8 (C-4,13), 46.2 (C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 72.2 (C-3), 122.1 (C-6), 141.2 (C-5) [20].



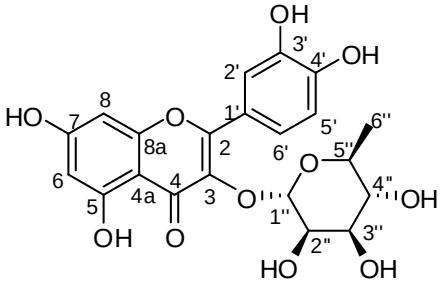
Scopoletin (1.10): pale yellow crystals, ^{1}H NMR (CDCl3) δ_H: 3.80 (3H, s, OCH_3-6), 6.10 (1H, d, *J* = 9.6 Hz, H-3), 6.70 (1H, s, H-8), 7.11 (1H, s, H-5), 7.75 (1H, d, *J* = 9.6 Hz, H-4); ^{13}C NMR (CDCl3) δ_C 56.8 (OCH_3-6), 104.0 (C-8), 110.0 (C-5), 112.56 (C-10), 112.62 (C-3), 146.1 (C-4), 147.1 (C-6), 151.4 (C-9), 152.9 (C-7), 164.0 (C-2) [21].



Kaempferol (1.11): yellow powder, ^{1}H NMR (Acetone-d_6): δ_H 8.16 (2H, d, J = 8.8, H-2',6'), 7.03 (2H, d, J = 8.8, H-3',5'), 6.54 (1H, d, J = 2.0, H-8), 6.27 (1H, d, J = 2.0, H-6); ^{13}C NMR (Acetone-d_6): δ_C 94.3 (C-8), 98.9 (C-6), 103.9 (C-10), 116.1 (C-3',5'), 123.1 (C-1'), 130.3 (C-2',6'),136.5 (C-3), 146.9 (C-2), 157.7 (C-5), 160.1 (C-9), 161.9 (C-4'), 165.0 (C-7), 176.5 (C-4) [22].

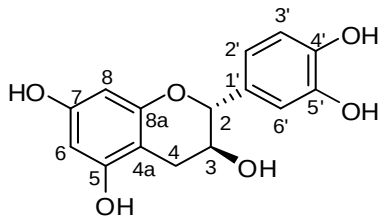


Afzelin (1.12): yellow powder, ^{1}H NMR (CD_3OD): δ_H 0.93 (3H, d, J = 5.4 Hz, H-6''), 3.29 (2H, m, H-4'', 5''), 3.68 (1H, dd, J = 3.3, 9.0 Hz, H-3''), 4.20 (1H, dd, J = 1.6, 3.3 Hz, H-2''), 5.34 (1H, d, J = 1.6 Hz, H-1''), 6.15 (1H, d, J = 2.4 Hz, H-6), 6.32 (1H, d, J = 2.4 Hz, H-8), 6.89 (2H, d, J = 8.4 Hz, H-3', 5'), 7.72 (2H, d, J = 8.4 Hz, H-2', 6'). ^{13}C NMR (CD_3OD): δ_C 16.47 (C-6''), 70.73 (C-5''), 70.83 (C-3''), 70.93 (C-2''), 72.01 (C-4''), 93.57 (C-8), 98.63 (C-6),102.31 (C-1''), 104.74 (C-10), 115.32 (C-3',5'), 121.45 (C-1'), 130.72 (C-2',-6'), 135.03 (C-3), 157.33 (C-2), 158.06 (C-9), 160.36 (C-4'), 162.00 (C-5), 164.64 (C-7), 178.40 (C-4) [23].



Quercitrin (1.13): yellow powder, ^{1}H NMR (CD_3OD): δ_H 0.93 (3H, d, J = 6.4 Hz, H-6''), 3.41 (1H, m, H-4''), 3.34 (1H, m, H-5''), 3.74 (1H, dd, J = 3.4, 9.4 Hz, H-3''), 5.34 (1H, d, J = 1.6Hz, H-1''), 4.21 (1H, dd, J = 1.6, 3.4 Hz, H-2''), 6.18 (1H, d, J = 2.0 Hz, H-6), 6.35 (1H, d, J = 2.0 Hz, H-8), 6.90 (1H, d, J = 8.4 Hz, H-5'), 7.29 (1H, dd, J = 2.0, 8.4 Hz, H-6'), 7.33 (1H, d, J = 2.0 Hz, H-2'). ^{13}C NMR (CD_3OD): δ_C 17.79 (C-6''). 72.04 (C-5''), 72.17 (C-3''), 72.26 (C-2''), 73.40 (C-4''), 94.85 (C-8), 99.94 (C-6), 103.67 (C-1''), 106.04 (C-10), 116.50 (C-2'), 117.08 (C-5'), 123.03 (C-1'), 123.12 (C-

6'), 136.38 (C-3), 146.53 (C-3'), 149.91 (C-4'), 158.64 (C-9), 159.44 (C-2), 165.97 (C-7), 163.33 (C-5), 179.77 (C-4) [23].



(+)-Catechin (1.14): yellow powder, ^{1}H NMR (CD_3OD): δ_H 2.51 (1H, dd, *J* = 16.2, 8.0 Hz, H-4b), 2.85 (1 H, dd, *J* = 16.2, 5.4 Hz, H-4a), 3.99 (1H, ddd, *J* = 8.0, 7.6, 5.4 Hz, H-3), 4.56 (1H, d, *J* = 7.6 Hz, H-2), 5.88 (1H, d, *J* = 2.2 Hz, H-6), 5.95 (1H, d, *J* = 2.2 Hz, H-8), 6.72 (1H, dd, J = 8.2, 1.6 Hz, H-6′), 6.77 (1H, d, *J* = 8.2 Hz, H-5′), 6.84 (1H, d, *J* = 1.6 Hz, H-2′); ^{13}C NMR (CD_3OD): δ_C 28.6 (C-4), 69.0 (C-3), 83.0 (C-2), 95.8 (C-8), 96.6 (C-6), 100.1 (C-10), 115.5 (C-4′), 116.4 (C-2′), 120.3 (C-6′), 132.4 (C-1′), 146.4 (C-3',5′),157.1 (C-9), 157.7 (C-7), 157.9 (C-5) [24].

1.3.2 Bioassay activity of isolated compounds

1.3.2.1 Cytotoxicity against KB and HeLa cell lines of isolated compounds

The cytotoxicity against HeLa and KB cell lines of all isolated compounds were determined using MTT colorimetric assay and the results were shown in Table 1.4

Table 1.4 *In vitro* cytotoxicity of compounds **1.1**-**1.14** against HeLa and KB cells.

Compound	IC_{50} (μg/mL)	
	KB	**HeLa**
Lupeol (**1.1**)	45.0	54.0
Betulin (**1.2**)	10.0	5.5
Betulinic aldehyde (**1.3**)	43.0	55.0
Betulinic acid (**1.4**)	83.0	10.0
Alphitolic acid (**1.5**)	38.0	28.0
Euscaphic acid (**1.6**)	53.0	95.0
Zizyberenalic acid (**1.7**)	9.5	13.0
A mixure of β-sitosterol (**1.8**) and stigmasterol (**1.9**)	>100	>100
Scopoletin (**1.10**)	55.0	97.0
Kaempferol (**1.11**)	88.0	>100
Afzelin (**1.12**)	>100	>100
Quercitrin (**1.13**)	>100	59.6
(+)-Catechin (**1.14**)	88.0	36.6
Adriamycin (standard)	0.018	0.018

$IC_{50} \leq 4$ = good activity

$4 < IC_{50} \leq 15$ = moderate activity

$15 < IC_{50} \leq 30$ = weak activity

$IC_{50} > 30$ = inactive

As seen in Table 1.4, all isolated compounds (**1.1-1.14**) were evaluated for their cytotoxicity against on KB and HeLa cells. Compounds **1.2** and **1.7** exhibited moderate cytotoxicity against both KB and HeLa cells, with IC_{50} values of 10.0, 5.5 µg/mL and 9.5, 13.0 µg/mL, respectively, while compound **1.4** showed only moderate cytotoxicity against on HeLa cells (IC_{50} = 10.0 µg/mL) and compound **1.5** showed only weak cytotoxicity against on HeLa cells (IC_{50} = 28.0 µg/mL). On the other hand, other compounds could be regarded as inactive.

1.4 Conclusion

In conclusion, the isolation and purification of the CH_2Cl_2 and MeOH crude extracts from the roots of *Zizyphus rugosa* Lam. gave nine triterpeniods; lupeol (**1.1**), betulin (**1.2**), betulinic aldehyde (**1.3**), betulinic acid (**1.4**), alphitolic acid (**1.5**), euscaphic acid (**1.6**), zizyberenalic acid (**1.7**), a mixure of *β*-sitosterol (**1.8**) and stigmasterol (**1.9**), one coumarin; scopoletin (**1.10**) and four flavonoids; kaempferol (**1.11**), afzelin (**1.12**), quercitrin (**1.13**) and (+)-catechin (**1.14**) (Figure 1.16). The chemical structures of all isolated compounds were characterized according to means of spectral analysis as well as comparison with the previous literature data.

All of the isolated compounds (**1.1-1.14**) were evaluated for their cytotoxicity against on KB and HeLa cells. Compounds **1.2** and **1.7** exhibited moderate cytotoxicity against both KB and HeLa cells, with IC_{50} values of 10.0, 5.5 µg/mL and 9.5, 13.0 µg/mL, respectively, while compound **1.4** showed only moderate cytotoxicity against on HeLa cells (IC_{50} = 10.0 µg/mL) and compound **1.5** showed only weak cytotoxicity against on HeLa cells (IC_{50} = 28.0 µg/mL). On the other hand, other compounds could be regarded as inactive. It is worth noting that compounds **1.2** and **1.7** might be lead compounds as antitumor agents.

The synthesis of isolated compounds and modification of their structures into novel active compounds is the future work for increasing quantity and biological activity that could be developed into new drugs. This will lead to better understanding on the interaction between active compounds and diseases.

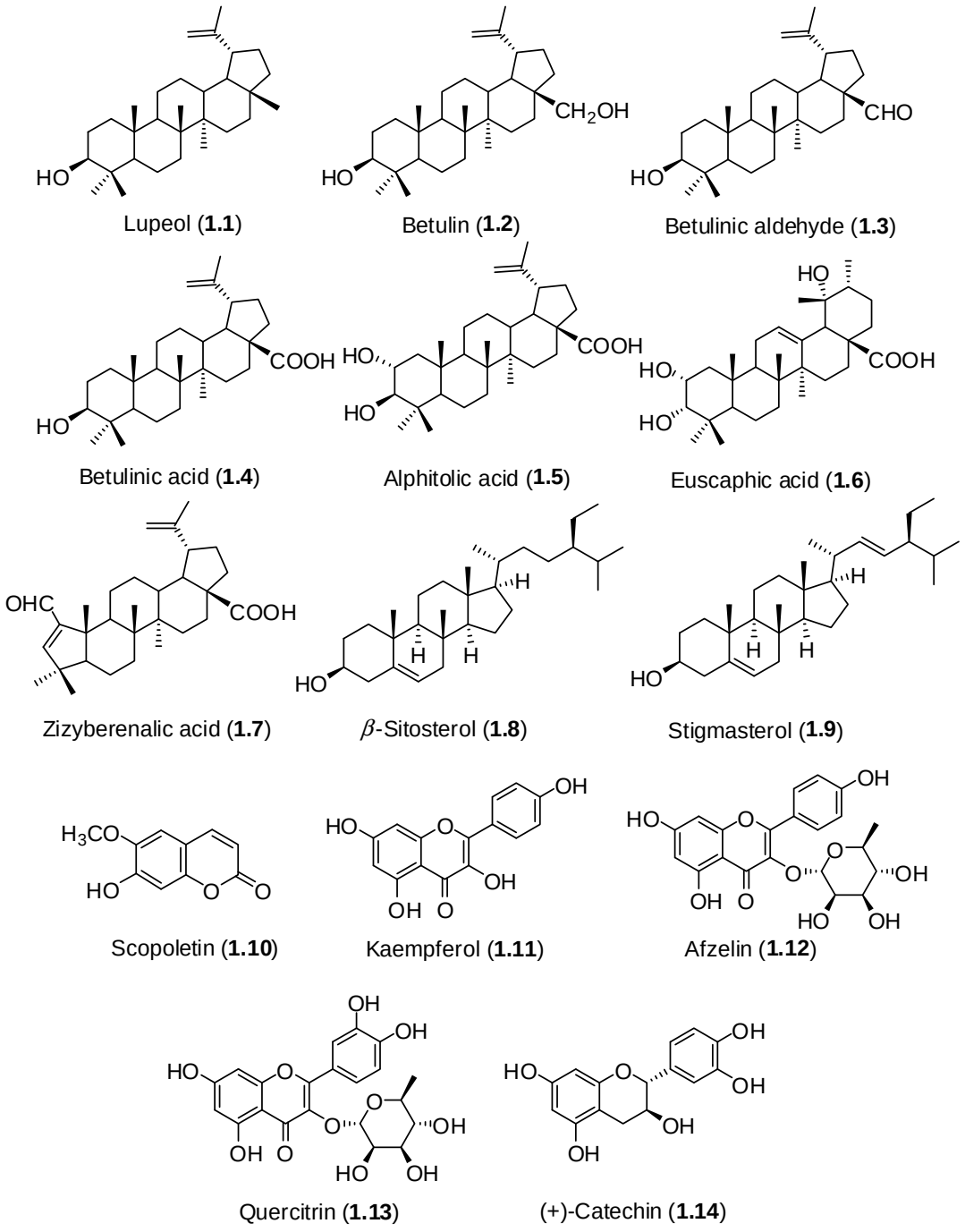


Figure 1.16 All of the isolated compounds (**1.1-1.14**) from the CH_2Cl_2 and MeOH crude extracts of *Zizyphus rugosa* Lam. roots.

CHAPTER II
CHEMICAL CONSTITUENTS FROM ROOTS OF *Antidesma Acidum* Retz.

2.1 Introduction

Natural products are metabolited produced by plants, fungi, bacteria, protozoans, and animals in response to external stimuli such as nutritional changes, infection and competition [25]. Reports of the use of medicinal plants go back to ancient times. Written records dated back at least 5000 years to the Sumerians, and archeological records suggest even earlier use of medicinal plants [26]. To date, acetylcholinesterase inhibitors as drugs from plants have been synthesized and the mode of binding for AChE inhibitors has also been determined such as rivastigmine, galantamine, hamayn, tacrine, donepezil, lobeline, sophoramine and cytosine (Figure 2.1) [27-33].

Many neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. As research progresses, many similarities appear which relate these diseases to one another on a sub-cellular level. Discovering these similarities offers hope for therapeutic advances that could ameliorate many diseases simultaneously. There are many parallels between different neurodegenerative disorders including atypical protein assemblies as well as induced cell death [34-35].

Alzheimer's disease (AD) is an acquired disorder of cognitive, behavioral impairment, primarily memory loss and in the later stages of the disease language deficits, depression, agitation, mood disturbances and psychosis with a long and progressive course. AD takes a significant toll on patients' families or caretakers and also has detrimental effects on the patient as well.

Acetylcholine (ACh) is the primary neurotransmitter that facilitates learning and increases attention. The ACh deficiency seen in AD led to the formulation of the cholinergic hypothesis, which states that the inability to transmit neurologic impulses across brain synapses is the cause of cognitive. Three major principles underlie the

use of cholinergic agents to treat AD. The first is reduced activity of choline acetyltransferase (ChAT) in the cerebral cortex. Levels of ChAT depletion correlate with the extent of neuritic plaque formation. Second, a reduction occurs in presynaptic muscarinic type 1 and nicotinic receptors but postsynaptic muscarinic type 2 receptors are preserved. Third, the large neurons responsible for the supply of ACh to the cerebral cortex, important for attention and new learning, are lost. Neuropathologic studies have shown the presence of NFTs in these neurons. In addition, cholinergic agonists have been found to facilitate the learning process, which also supports the important role of ACh in attention and learning. Among the different types of drugs that are used to modify cholinergic neurotransmission, only the cholinesterase (ChE) inhibitors have been effective to date. Physostigmine, rivastigmine and galanthamine are unique ChE inhibitors that have been approved by the US Food and Drug Administration for the treatment of mild to moderate AD [36-38].

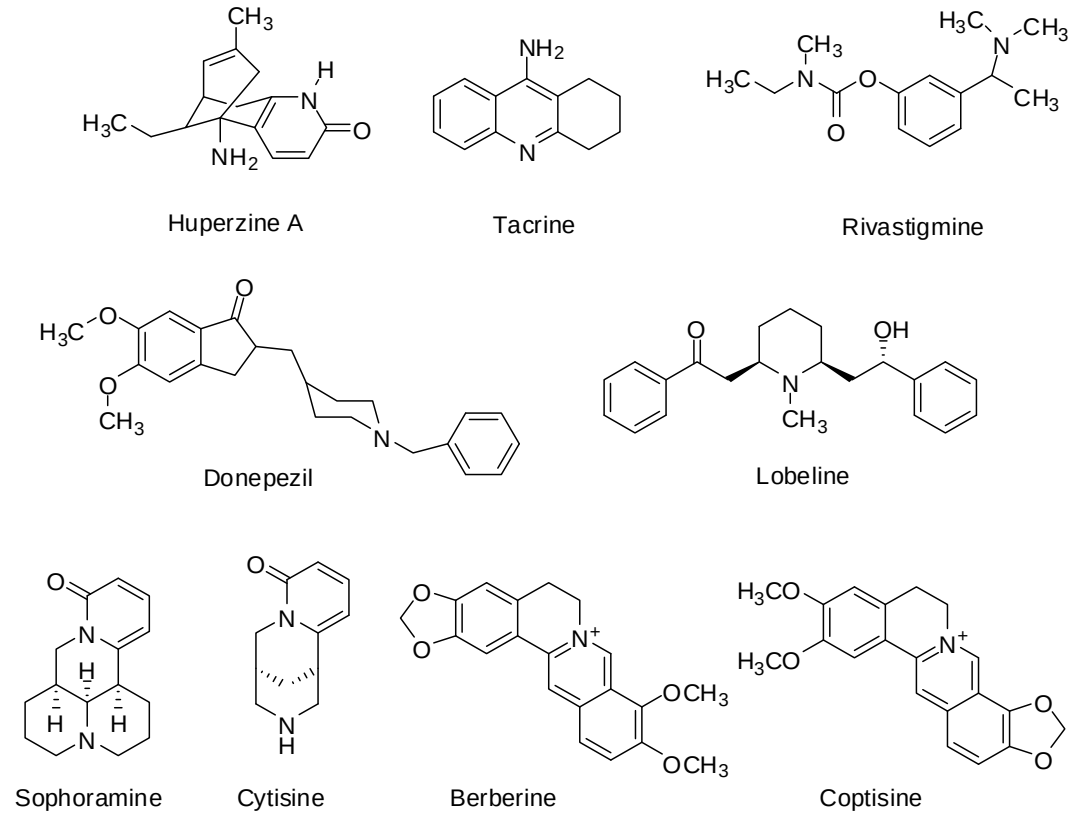


Figure 2.1 Acetylcholinesterase inhibitors as drugs from plants.

Although several inhibitors have attracted the attention of neuropharmacologists as AChE inhibitors from plant, the potential effectiveness offered is limited by the appearance of central and peripheral side effect and their toxic properties. Recently, researchers are interest for searching in the other natural inhibitory compounds and are not toxic to AD patients.

Plants in genus *Antidesma* (Euphorbiaceae) are known to produce several types of compounds having some significant biological activities. One of these types is coumarins type that showed good activity as AChE inhibitor [39]. As a part of searching for natural inhibitors for AChE and there are no report on AChE inhibitory activity from this genus that contains coumarin compounds. Therefore, the examination in the efficient constitution from this genus is concern.

2.1.1 Biosynthesis and Phytochemistry of *Antidesma* genus

Many coumarins have been isolated from *Antidesma* (Euphorbiaceae). The pathway of coumarin biosynthesis has been largely outlined during the ‘60s and ‘70s, with the help of tracer feeding experiments. Radiolabeled cinnamic acid was incorporated into coumarin and 7-hydroxycoumarins. Other tracer experiments conducted with *Lavandula officinalis*, a plant that produces coumarin as well as 7-hydroxylated coumarins, revealed that in the latter instance *para*-hydroxylation preceded the *ortho*-hydroxylation required for lactonization). This indicated that umbelliferone is derived from *cis*-*p*-coumaric acid, whereas coumarin originates from *cis*-cinnamic acid), and may imply different enzymes for the *ortho*-hydroxylation/lactonization of coumarin versus umbelliferone.

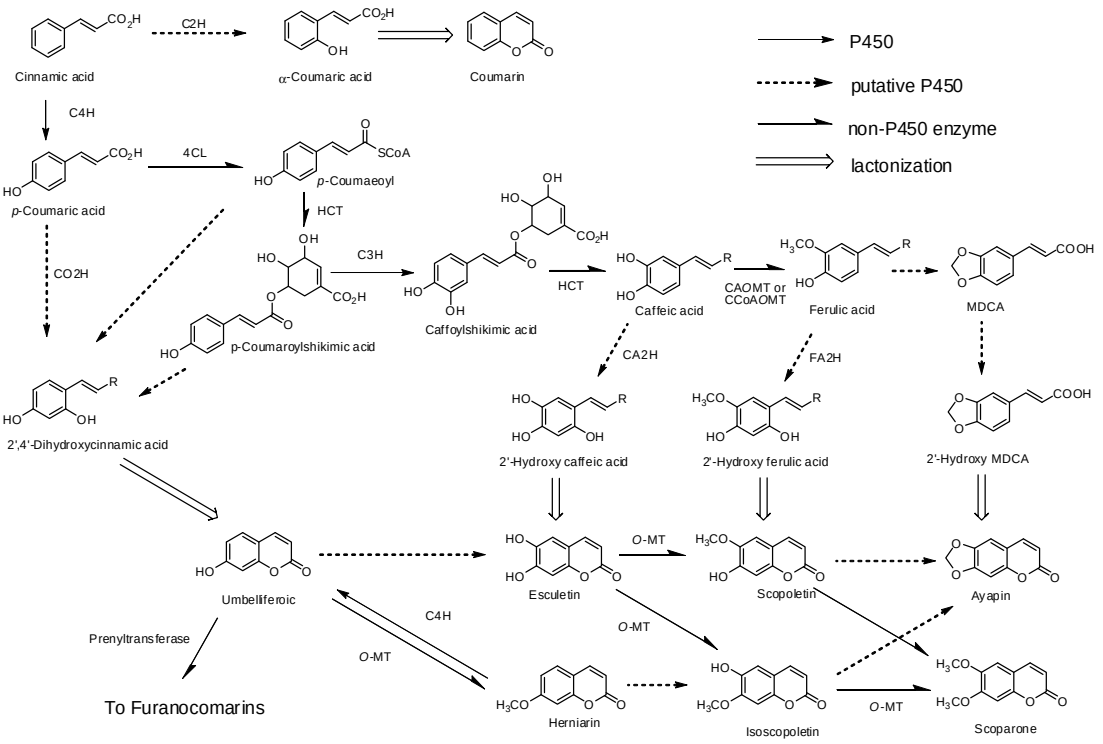


Figure 2.2 Biosynthetic pathways of coumarins and its derivatives.

Phenylpropanoid pathway leading to coumarins pathways have been unequivocally established. Glycosylated compounds are not shown for clarity. Enzymes assigned by a question mark are hypothetical. R = CO_2H or CO-SCoA C2H, cinnamic acid 2-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CO2H, 4-coumaric acid 2-hydroxylase; HCT, hydroxycinnamoyl-transferase; CA*O*MT, caffeic acid *O*-methyltransferase; CCoA*O*MT, caffeoyl CoA *O*-methyltransferase, CA2H, caffeic acid 2-hydroxylase; FA2H, ferulic acid 2-hydroxylase; MDCA2H, methylenedioxycinnamic acid 2-hydroxylase; *O*-MT, *O*-methyltransferase The *ortho*-hydroxylation is a key step of coumarin biosynthesis, that has received insufficient attention. In initial experiments, double-labeled (*ortho*-^{3}H, *ring*-1-^{14}C) cinnamic acid was fed to *Melilotus alba* shoots or *Gaultheria procumbens* leaves, and the retention of label was monitored upon conversion to *o*-coumaric acid . An NIH shift was proposed because of insignificant decrease of the ^{3}H:^{14}C ratio, which is an indication of a cytochrome P450 monooxygenase reaction mechanism. A following report addressed the formation of coumarin with extracts from *Melilotus alba*, a plant that produces high levels of

coumarin. This study allocated the *ortho*-hydroxylation of cinnamic acid to the chloroplast and again suggested a P450-dependent hydroxylation mechanism. Unfortunately, the in *vitro* results could not be reproduced, and the classes of the enzyme involved as well as its subcellular site remain to be established. As revealed later, the early experiments may have suffered from fundamental analytical problems, since the chromatography and recrystallization techniques employed were likely insufficient to separate the various cinnamic acids. Nevertheless, the proposed conversion of cinnamic to *o*-coumaric acid received some support by precursor feeding studies done with *Petunia* chloroplasts, which ascribed cinnamate 2-hydroxylase, including the formation of coumarin, and lack of cinnamate 4-hydroxylase to these organelles. In light of the studies done since with *Ammi majus* microsomes on the biosynthesis of furanocoumarins it appears possible that the '*ortho*-hydroxylase' is an exceptionally labile CYP enzyme, in contrast to the CYPs hydroxylating cinnamic acids in *para*- or *meta*-position. Overall, the *ortho*-hydroxylation of cinnamic (or 4-coumaric) acid, being of pivotal importance for all coumarins, remains a missing link in the network of phenylpropanoid biosynthesis [40].

2.1.2 Chemical constituents from *Antidesma* species and their biological activities

There are many chemical and biological investigations which have not been carried out on *Antidesma* genus. The coumarin components of this genus were previously reported. In addition, other compounds have demonstrated a number of interesting biological benefits, including antioxidant, antiinflammatory, antiapoptotic and anticarcinogenic activities [41].

A. montana Bl. is a small tree ca 12 m in height which occurs in Southern China, Peninsular Malaysia and Indonesia. This plant is a wild plant well-known there for its edible fruit and traditional medicinal value of the leaves. Cyclopeptide alkaloids, myrianthine B, aralionine B and dihydro AM-l (Figure 2.2) were isolated from the leaves and terminal branches of this plant [42].

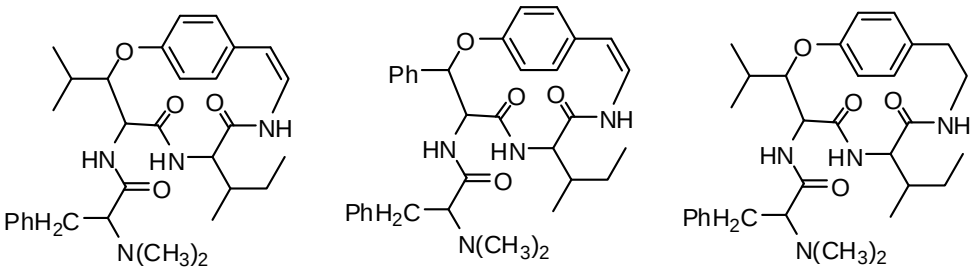
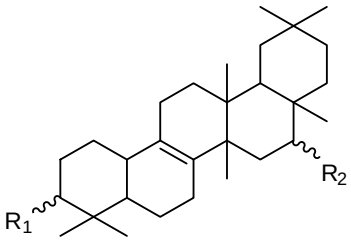


Figure 2.3 Isolated compounds from *A. montana* Bl.

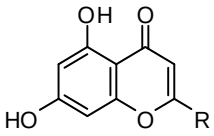
A. menasu Miq. ex. is a shrub or small tree. Isomuhiflorene and its derivatives (Figure 2.3) have been isolated from the aerial parts of *A. menasu.* Both of these compounds displayed diuretic activity in experimental animals [43].



	R_1	R_2
16α-Hydroxy-3-ketoisomultiflorene	=O	α-OH, β-H
16α-Acetoxy-3-ketoisomultiflorene	=O	α-OAc, β-H
16-Ketoisomultiflorene	H	=O
3, 16-Diketoisomultiflorene	=O	=O
3α,16α-Dihydroxyisomultiflorene	α-OH, β-H	α-OH, β-H
3β-Hydroxy-16-ketoisomultiflorene	β-OH, α-H	=O
3β-Acetoxy-16-ketoisomultiflorene	β-OAc, α-H	=O
Isomultiflorene	β-OH, α-H	H

Figure 2.4 Isolated compounds from *A. menasu* Miq.

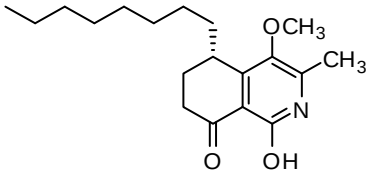
A. membranaceum Mtill. is a shrub or small tree occurring in equatorial Africa. 5,7-Dihydroxy-2-nonadecyl-cromone, 5,7-dihydroxy-2-eicosyl-cromone, 5,7-dihydro xy-2-heneicosyl-cromone and tetrahydroisoquinoline alkaloid and antidesmone (Figure 2.4) were isolated from this plant [44].



5,7-Dihydroxy-2-nonadecyl-chromone R = $C_{19}H_{39}$

5,7-Dihydroxy-2-eicosyl-chromone R = $C_{20}H_{41}$

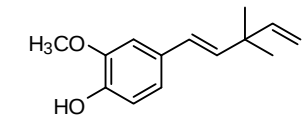
5,7-Dihydroxy-2-heneicosyl-chromone R = $C_{21}H_{43}$



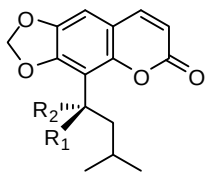
Antidesmone

Figure 2.5 Isolated compounds from *A. membranaceum* Mtill.

A. pentandrum Merr. var. *barbatum* (Presl) Merr. is a small shrub distributed throughout the Ryukyus, the northern Philippines, and Taiwan, and tannin constituents from the leaves of this plant have been reported. Twenty one compounds (Figure 2.5) were isolated from the roots of this plant [45].

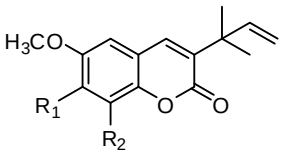


Antidesmol



Antidesnone R_1+R_2 = O

Antidesnol R_1 = OH, R_2 = H

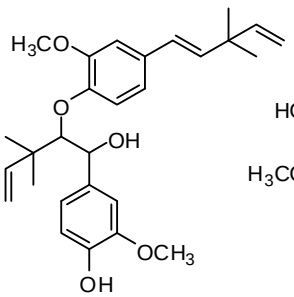


Barbatumols A R_1 = OCH_3, R_2 = OH

Barbatumol B R_1 = OH, R_2 = OCH_3

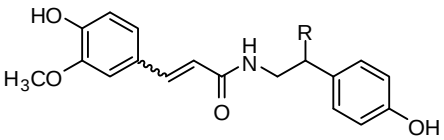
3-(1,1-Dimethylallyl)-scopoletin R_1 = OH, R_2 = H

Obtusidin R_1 = R_2 = OH



Antidesmanins E, *erythro*

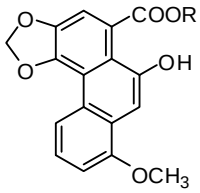
Antidesmanins F, *threo*



N-trans-feruloyltyramine R = H

N-cis-feruloyltyramine R = H

N-trans-feruloctopamine R = OH



Sodium aristolochate-I R = Na

Aristolochic acid I methyl ester R = CH_3

Figure 2.6 Isolated compounds from *A. pentandrum* Merr. var. *barbatum* (Presl) Merr.

2.1.3 Botanical aspect and distribution

Euphorbiaceae are herbs, shrubs or trees, seldom climbers; monoecious or dioecious; stems sometimes spiny or succulent and cactus-like; with or without coloured or white latex. *Indumentum* absent or of simple, urticating (stinging), stellate and/or lepidote hairs especially on the lower leaf surface and in the inflorescences. *Stipules* usually present, free, rarely connate, persistent or usually caducous, sometimes glandular. *Leaves* alternate or opposite (or pseudo-whorled), simple or seldom trifoliolate, sometimes reduced to scales, usually petiolate, base often with two glands, margin entire to variously crenate, dentate or serrate, teeth often glandular, sometimes palmately lobed; venation pinnate or basally palmate. *Inflorescences* cauliflorous, to usually axillary or terminal, cymose-derived, but very variable in form: spikes, racemes, cymes, thyrses, panicles, solitary flowers, faciculate flowers or pseudo-flowers (cyathia in *Euphorbia* and *Pedilanthus*), unisexual or bisexual, the bisexual usually with the pistillate flowers proximal and the more numerous staminate flowers distal; bracts present, often basally glandular, with a single (usually pistillate) or several (usually staminate) flowers; bracteoles absent or indistinct to 2 inside the bract to several. *Flowers* unisexual (seldom bisexual), usually actinomorphic, pedicellate or sessile; sepals usually 3 or 5, seldom absent, free or partly connate, valvate or imbricate; petals usually absent (especially in pistillate flowers) or usually 5, smaller or longer than sepals; disc usually present, annular or of separate lobes, usually outside the stamens or ovary. *Staminate flowers*: stamens 1 to usually many, filaments free to variously united; anthers with 2-4 thecae, opening with longitudinal slits; pistillode present or absent. *Pistillate flowers*: staminodes seldom present; ovary superior, 1- to many-locular, usually 3-locular; ovules 1 or 2 in each locule, apically attached to the central column; style absent or present; stigmas usually as many as locules, lobed (or disciform), apically often partly to completely split, often with long (branched) papillae on upper surface. *Fruits* usually capsules (schizocarps or rhegmas), sometimes indehiscent drupes or berries (transitions exist: tardily splitting fleshy capsules), dehiscense usually septicidally and loculicidally, sometimes one fruit only partly so, leaving bivalved segments, columella persistent. *Seeds* usually 3 or 6, often not all developing, smooth (to warty), naked or with an

apical fleshy caruncle or partly to completely covered by an aril; endosperm usually copious and oily.

The family occurs worldwide except in the polar and subpolar regions, the great majority of species in tropical and subtropical regions, with ca. 300 genera and ca. 5,000 species. Eighty-seven genera and ca. 425 species in Thailand [46].

Antidesma is a genus of tropical plant in the Euphorbiaceae. This is a variable plant which may be short and shrubby or tall and erect, approaching 30 meters in height.It has large oval shaped leathery evergreen leaves up to about 20 centimeters long and seven wide. The flowers have a strong, somewhat unpleasant scent. The staminate flowers are arranged in small bunches and the pistillate flowers grow on long racemes which will become the long strands of fruit. The fruits are spherical and just under a centimeter wide, hanging singly or paired in long, heavy bunches. They are white when immature and gradually turn red, then black.When they are still white they have sour and astringent taste, sour taste when they are red and have sweet and sour taste when they are black. Antidesma is native to the Old World Tropics. They have about 100 species and the highest number in South-East Asia. There are about 12 species are native to Thailand, for example [47].

A. acidum Retz. (มะเม่าสร้อย)

A. bunius Spreng. (มะเม่าดง)

A. ghaesembilla Gaerth. (เม่าไข่ปลา)

A. leucocladon Hook. f. (ผักหวานหลังขาว)

A. leucopodum Miq. (เม่าโปโล)

A. martabanicum Presl (ส้มเม่าเขา)

A. microphyllum Hemsl. (ตะไคร้น้ำ)

A. montanum Bl. (มะเม่าขน)

A. neurocarpum Miq. (พลองขาว)

A. sootepense Craib. (มะเม่าสาย)

A. thwaitesianum Muell. Arg (เม่าหลวง)

A. velutinosum Bl. (มะเม่าควาย)

A. acidum Retz. is a large deciduous shrub or small tree. Shoots rusty-tomentose. Petioles 2-5 mm long. Leaves elliptic-obovate to oblong-oblanceolate, 4-

12 x 3-5 cm, acuminate, cuneate at the base, lateral nerves 6-8 pairs, faint, glabrous above, sparingly pubescent usually only along the midrib and main nerves beneath. Stipules linear-lanceolate, 4-5 mm long, acute, pubescent. Inflorescences terminal, simple or with 1-2 basal branches, up to 8 cm long; axis usually glabrous; bracts ovate, 0.5 mm long, chaffy. Male flowers: pedicels 1-1.5 mm long, slender; calyx-lobes 4, broadly ovate, 0.5 mm long, acute, glabrous without, pilose within; disc of 2(-3) connate pulviniform glands; stamens 2(-3), arising out of the centre of each gland, filaments 2 mm long, anthers 0.5 mm long; pistillode 0. Female flowers: pedicel 0.3 mm long, stout; calyx ± as in the ♂; disc annular, 0.8 mm diam.; ovary ellipsoid,0.8 x 0.5 mm, smooth, glabrous; styles 2, terminal, bifid, 0.5 mm long. Fruit subglobose when fresh, ovoid when dried, 3.5-4 x 2.5 mm, reddish-purple; endocarp 4-ridged, coarsely reticulate (Figure 2.6) [48].

Figure 2.7 The stems, roots, fruits and flowers of *A. acidum* Retz.

2.1.4 Biological activities

2.1.4.1 Anticholinesterase inhibitory activity

Alzheimer's disease (AD) is a progressive degenerative neurological disorder resulting in impaired memory and cognitive function. In AD patients, there are decreased levels of acethylcholine in the brain areas related to memory and learning. The primary approach in treating AD has aimed at augmenting the cholinergic system. The process begins when acetylcholine is released to travel across the synaptic cleft where it binds to a receptor on the other side of the synapse (post-synaptic terminal). Once the signal is triggered acetylcholine is rapidly broken down by an enzyme, acetylcholinesterase (AChE), and liberating choline. This is followed by a rapid hydrolysis of the acylated enzyme yielding acetic acid and the restoration of the esteratic cells. In spite of the multi-factorial nature of AD, most current agents follow one therapeutic approach, based on the so-called cholinergic hypothesis of cognitive dysfunction. This hypothesis postulates that at least some of the cognitive decline experienced by patients of AD results from a deficiency in neurotransmitter acetylcholine and thus in cholinergic neurotransmission, which seems to play a fundamental role in memory. In late stages of AD, levels of AChE decline by up to 85% and butyrylcholinesterase (BuChE) represents the predominant cholinesterase in the brain. Such studies have targeted BuChE as a new approach to intercede in the progression of AD.

From the literature review on the chemical constituents and their biological activities of plants in Euphorbiaceae family and no report on chemical constituents and biological activity on *A.acidum* Retz, this plant was selected for further investigation.

The objectives of this research:

The main objectives in this investigation are as follows:

1. To isolate and purify compounds from the roots of *A. acidum* Retz.
2. To identify the chemical structures of all isolated compounds.
3. To evaluate the cytotoxicity against HeLa and KB cell lines of the isolated compounds.
4. To evaluate the AChE and BuChE inhibitory activity of the isolated compounds.

2.2 Experimental

2.2.1 Plant material

The roots of *A. acidum* Retz. were collected from Kalasin Province of Thailand in October, 20011 and identified by Ms. Suttira Khumkratok, a botanist at the Walai Rukhavej Botanical Research Institute, Mahasarakham University, where a voucher specimen (Khumkratok no.1-12) was deposited.

2.2.2 General experimental procedures

Melting points were determined on a Fisher-John apparatus and was uncorrected. HRESIMS were obtained by Micromass LCT mass spectrometers. UV spectra were taken on a UV-160A spectrometer (SHIMADZU). NMR spectra were recorded with a Varian model Mercury^{+} 400 spectrometer operated at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR and a Bruker 400 AVANCE spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvent and using TMS as an internal standard in some cases. Most solvents used in this research were commercial grade and were distilled prior to use. Absorbents such as Dianion HP-20, Sephadex LH-20 and silica gel 60 Merck cat. No. 7730, 7734 and 7749 were used for quick column chromatography, open column chromatography and centrifugal thin layer chromatograph (Chromatotron), respectively. Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.2.3 Extraction and purification

The air-dried roots of *A. acidum* Retz. (6.5 kg) were collected in October 2011 from Kalasin Province, Thailand, and successively extracted with CH_2Cl_2 and MeOH (each 500 mL, 16 h) at room temperature. The solvents were evaporated under vacuum to yield CH_2Cl_2 (40.2 g) and MeOH (80.3 g) crude extracts. The CH_2Cl_2 crude extract was subjected to silica gel column chromatography (Merck Art 7730) using hexane and CH_2Cl_2 as eluents with increasing polarity to afford seven major fractions (AD1-AD7). The fraction AD3 was chromatographed on silica gel column

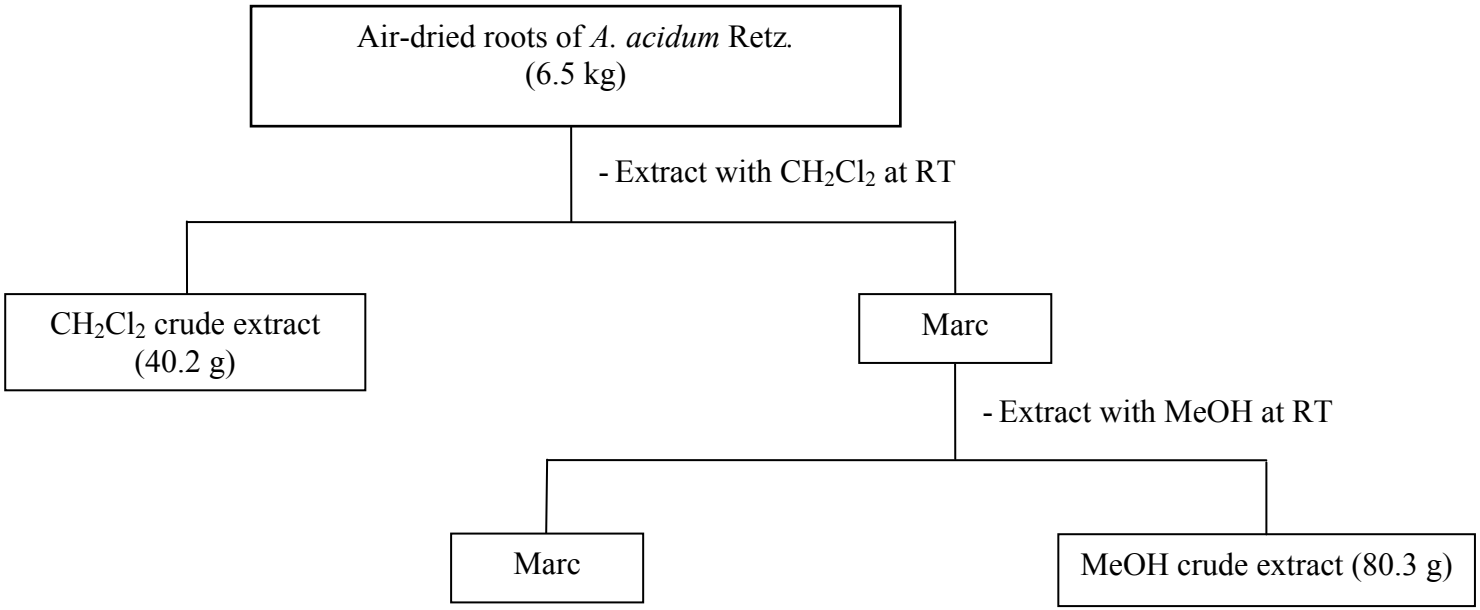
using a stepwise gradient elution of CH_2Cl_2 and hexane to give three fractions (AD3-1-AD3-3). Fraction AD3-2 was further purified by centrifugal thin layer chromatograph (Chromatotron) using a stepwise gradient elution of hexane and EtOAc to yield antidesmol (**2.1**, 2.0 mg). The fraction AD4 was chromatographed on silica gel column using CH_2Cl_2 and hexane as a stepwise gradient eluents to give three fractions (AD4-1-AD4-3). Then fraction AD4-3 was purified by Chromatotron using a stepwise gradient elution of hexane and EtOAc to obtain corylifolin (**2.2**, 5.6 mg). The fraction AD5 was chromatographed on silica gel column using a stepwise gradient elution of EtOAc in hexane to yield mellein (**2.3**, 20.8 mg) and a mixture of β–stitosterol (**2.4**) and stigmasterol (**2.5**) (34.5 mg). The fraction AD6 was subjected to silica gel column chromatography using EtOAc and hexane as eluents to afford three fractions (AD6-1-AD6-3). Fraction AD6-1 was loaded on Chromatotron with increasing polarity of EtOAc and hexane to afford 5-cholesten-3β-ol (**2.6**, 30.5 mg) and 4-cholesten-3-one (**2.7**, 35.6 mg). Fraction AD6-3 was purified by Sephadex LH-20 using 20% MeOH in CH_2Cl_2 to obtain 3-(1,1-dimethylallyl)-scopoletin (**2.8**, 20.3 mg) and 5,7-Dihydroxy-2-eicosyl-cromone (**2.9**, 40.1 mg). The fraction AD7 was load on silica gel column chromatography using hexane, EtOAc and MeOH as a gradient eluents to give two fractions (AD7-1-AD7-2). Fraction AD7-1 was subjected to Chromatotron with the stepwise gradient elution of EtOAc and hexane to afford 2,5-dimethoxy-1,4-bezoquinone (**2.10**, 20.5 mg) and barbatumol A (**2.11**, 50.6 mg). Fraction AD7-2 was subjected to Sepadex LH-20 with MeOH and CH_2Cl_2 as eluents to yield *N-trans*-feruloyltyramine (**2.12**, 60.3 mg) and syringic aldehyde (**2.13**, 10.1 mg) (Figure 2.1).

Finally, the MeOH crude extract was dissolved in water and the aqueous layer was cyclic loaded on Dianion HP-20 column with increasing amount of water. Water was washed through the column to remove any sugar and salt, and the organic material was eluted using 100% MeOH. This MeOH eluent was subject to silica gel column chromatography, using EtOAc-MeOH-H_2O (9:1:0, 40:10:1, 70:30:3, respectively) to yield three major fractions AM1-AM3. The fraction AM1 was purified by Sephadex LH-20 using 50-100% MeOH in EtOAc as an eluent and then was subjected to Chromatotron with the stepwise elution of EtOAc and hexane to obtain *p*-hydroxybenzoic acid (**2.14**, 118 mg). The fraction AM2 was purified by

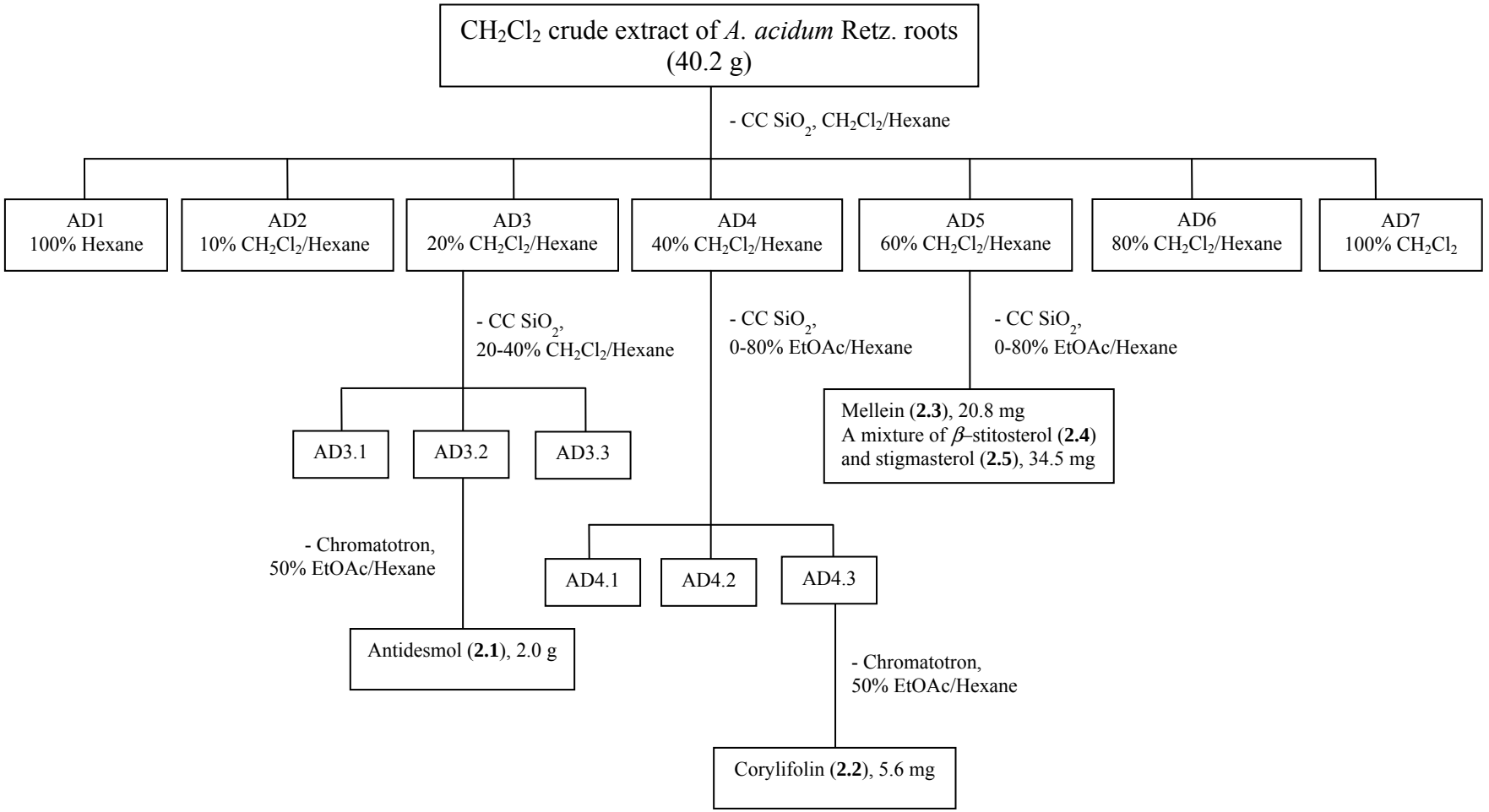
Sephadex LH-20 using 100% MeOH as an eluent and then was subjected to Chromatotron and eluted with the gradient eluents of EtOAc and hexane to obtain taxifolin (**2.15**, 40.3 mg), (+)-catechin (**2.16**, 60.2 mg) and (-)-Gallocatechin (**2.17**, 45.9 mg) (Figure 2.2).

The identification of all isolated compounds was determined by means of various spectroscopic methods including MS, 1D and 2D NMR techniques as well as comparison with the previous literature data.

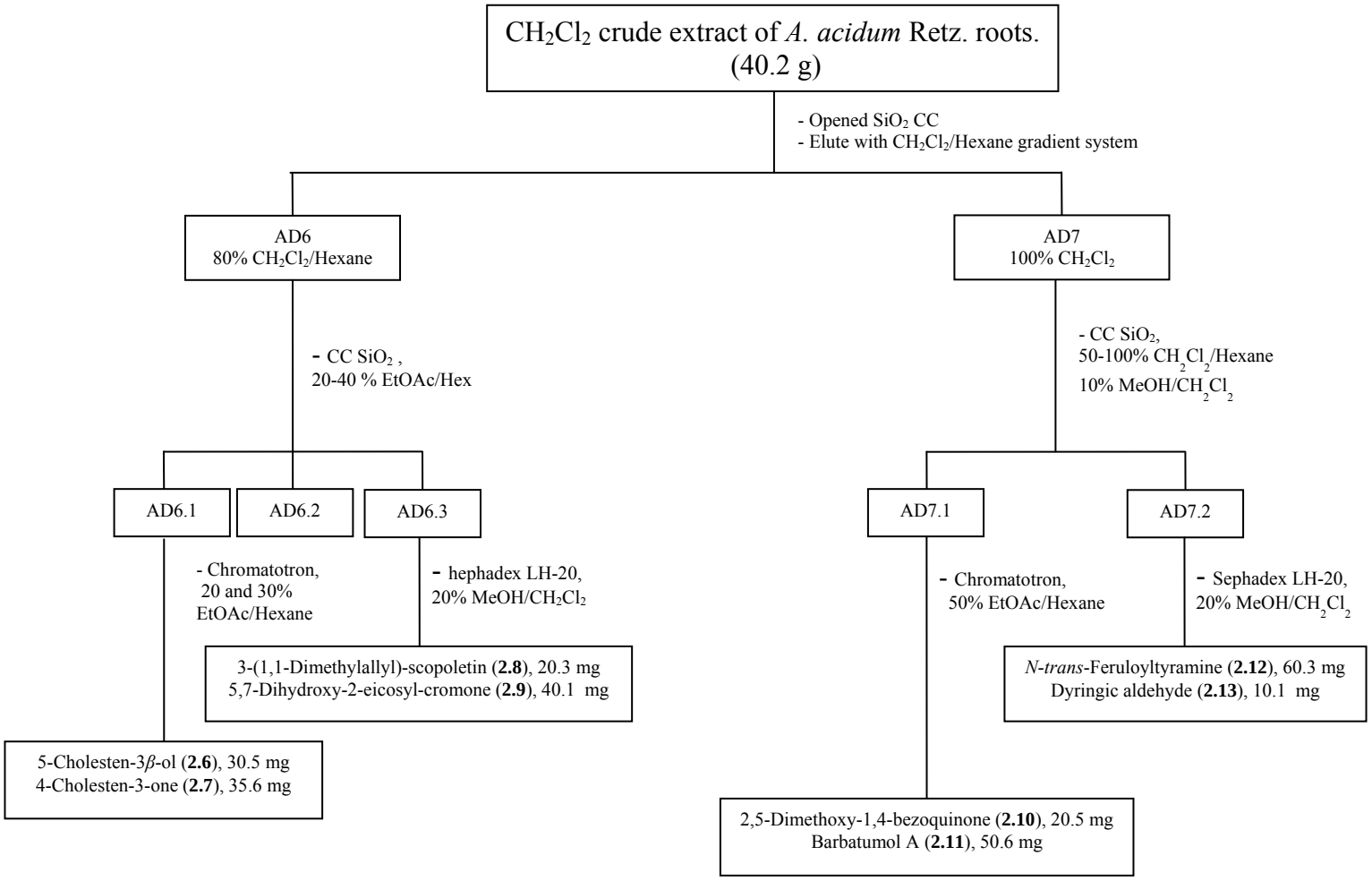
The isolation and purification of all isolated compounds from the CH_2Cl_2 and MeOH crude extracts of the roots of *A. acidum* Retz. were briefly summarized in Schemes 1.1-1.3.



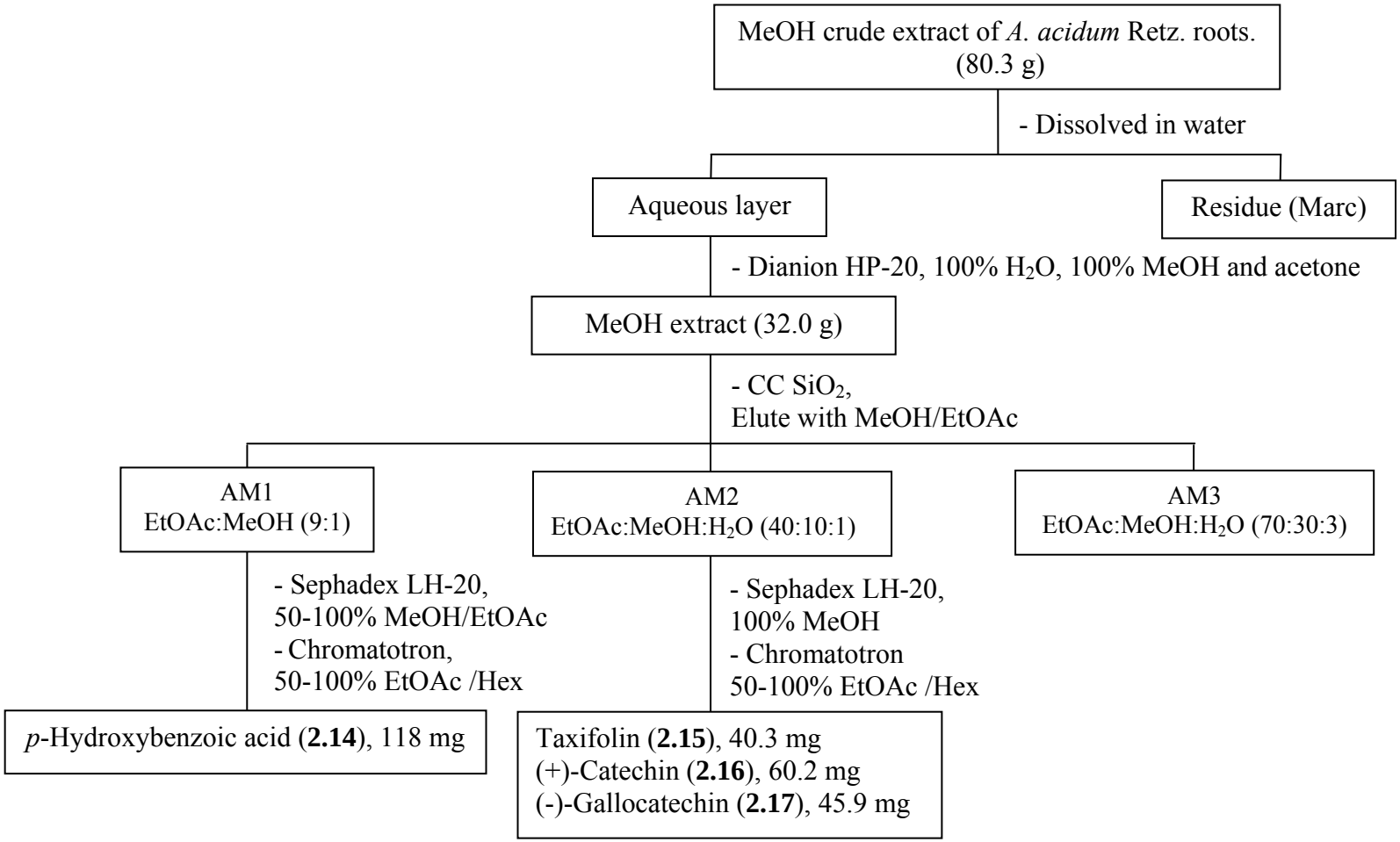
Scheme 2.1 Extraction procedure of *A. acidum* Retz.



Scheme 2.2 Isolation procedure of the CH_2Cl_2 crude extract of *A. acidum* Retz. roots.



Scheme 2.3 Isolation procedure of the CH_2Cl_2 crude extract of *A. acidum* Retz. roots. (cont.)



Scheme 2.4 Isolation procedure of the MeOH crude extract of *A. acidum* Retz. roots.

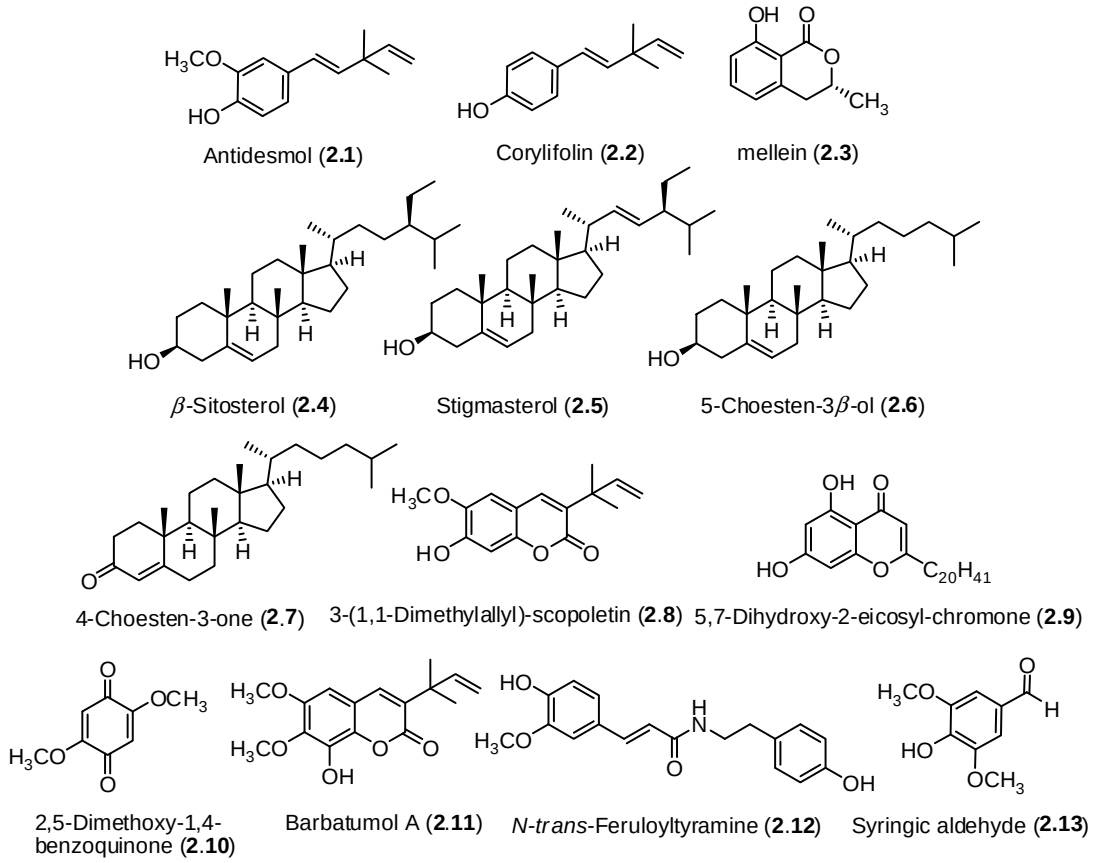


Figure 2.8 Isolated compounds from the CH_2Cl_2 crude extract of *A. acidum* Retz. roots.

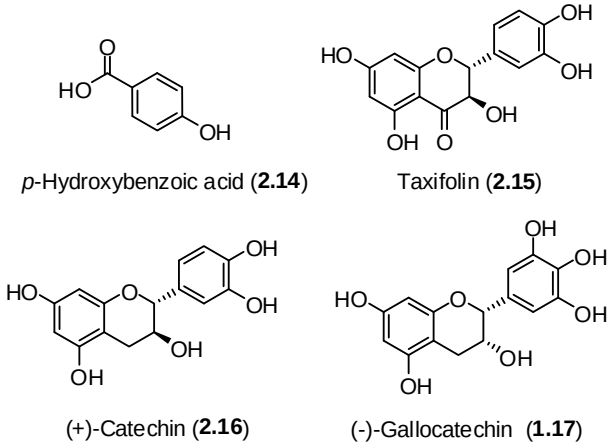


Figure 2.9 Isolated compounds from the MeOH crude extract of *A. acidum* Retz. roots.

2.2.4 Bioassay procedure

2.2.4.1 The AChE and BuChE Activities Assay

This assay was performed base on Ellman's method with some modification [49]. The test relies on the cleavage by cholinesterase of acetylthiocholine to form thiocholine which in turn reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to give a yellow colored 2-nitrobenzoate-5-mercaptothiocholine (Figure 2.9). Inhibitors which may be naturally occurring or synthetic molecules decrease enzyme activity or prevent any substraste molecules from reacting with the enzyme.

- Chemical reagents

All commercial solvents used in this research were distilled prior to use except for those which were reagent grades.

Acetylcholinesterase (AChE) from electric eels (Type-VI-S lypophilized powder, EC 3.1.1.7), butyrylcholinesterase (BuChE) from horse serum (lypophilized powder EC 3.1.1.8), acetylthiocholine iodide (ATCl), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), galanthamine and 2,2-diphenyl-1-picryhydrazyl (DPPH) radical were purchased from Sigma (St. Louis, MO, USA). *Tris*-(hydroxymethyl)-aminomethane (*Tris*-HCl) was purchased from Merck (Darmstadt, Germany).

- Chemical preparation

- **Buffers**: The following buffers were used; buffer A:50 mM *Tris*–HCl, pH 8; buffer B: 50 mM *Tris*–HCl, pH 8 containing 0.1% bovine serum albumin (BSA); buffer C: 50 mM *Tris*–HCl, pH 8 containing 1 M NaCl and 0.2 mM $MgCl_2.6H_2O$.

- **Enzymes**: Cholinesterase enzymes (AChE and BuChE) were dissolved in buffer A to make 1130 U/ml stock solution, and further diluted with buffer B to get 0.28 U/ml enzymes for the microplate assay, or diluted with buffer A to get 3 U/ml enzyme for the TLC assay.

- **Substrate**: 5 mM ATCI in buffer A was used for the TLC assay and 1.5 mM in MilliQ water was used for the microplate assay.

- **Ellman reagent:** 5 mM DTNB in buffer A was used for the TLC assay and 3 mM DTNB in buffer C was used for the microplate assay.

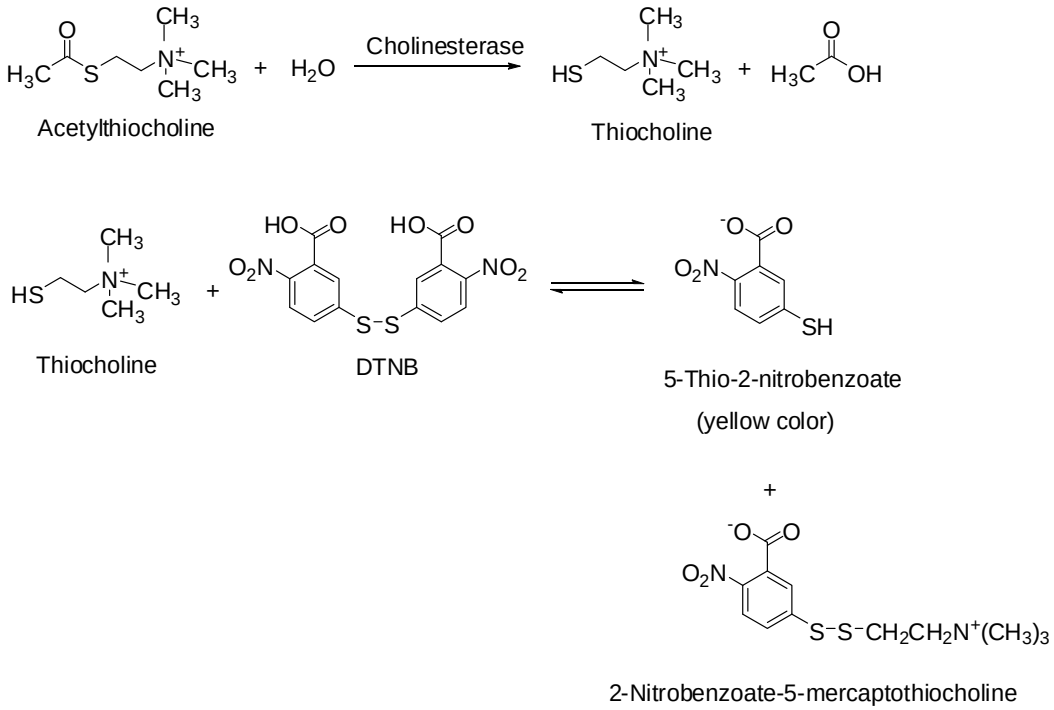


Figure 2.10 Cholinesterase catalyzed hydrolysis of acetylthiocholine.

2.2.4.2 Cholinesterase inhibitory method

AChE and BuChE inhibitory activities were measured by slightly modifying the spectrophotometric technique based on Ellman's method [50]. In the 96-well plates, 25 mL of 1.5 mM ATCI in water, 125 mL of 3 mM DTNB in buffer C, 50 mL of buffer B, 25 mL of sample (1 mg/ml in MeOH diluted ten times with buffer A to give a concentration of 0.1 mg/mL), 25 mL of 0.28 U/mL of enzyme were mixed together and the absorbance was then measured at 415 nm over 2 min with a 5-s interval. The resulting velocity was calculated and used for the determination of the enzyme inhibitory activities.

$$\% \text{ inhibition} = \left(\frac{\text{Rate of blank - Rate of sample}}{\text{Rate of Blank}} \right) \times 100$$

2.3 Results and Discussion

2.3.1 Properties and structural elucidation of isolated compounds

2.3.1.1 Corylifolin (2.2)

Corylifolin (**2.2**): was obtained as white solid. Its molecular formula was established as $C_{13}H_{16}O$ by HREIMS. The ^{1}H NMR spectrum (Table 2.1) of **2.2** showed the presence of a *p*-hydroxyphenyl group, a 1,1-dimethylallyl group, and two *trans*-olefinic protons. The HMBC spectrum of **2.2** showed the correlations of H-2 and 6 (7.25, d, J = 8.5 Hz) with C-3, C-4, C-7 and H-3 and 5 (6.78, d, J = 8.5 Hz) with C-1, C-3, C-4, which confirmed the presence of *para* substitute phenol. The correlation of H-8 (6.09, d, J = 16.5 Hz) with C-1, C-2, C-6, C-7, C-10, C-12 C-13 and CH_3-12 and 13 (1.19, s) with C-7, C-8, C-9, C-10, C-11 revealed the existence of 1,1-dimethylallyl group. According to the above data, the structure of **2.2** was elucidated as a new natural compound or synthetically known compound 4-((*E*)-3,3-dimethylpenta-1,4-dienyl)-phenol.

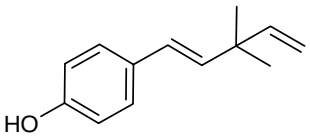


Figure 2.11 Structure of corylifolin (**2.2**, a new natural compound).

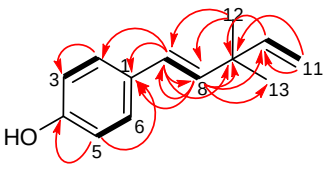
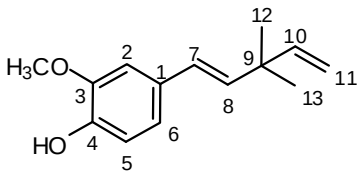


Figure 2.12 Selected HMBC (arrow curves) and COSY (bold lines) correlations of **2.2**.

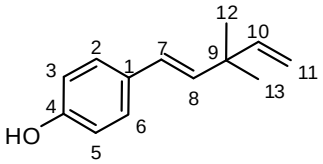
Table 2.1 ^{1}H, ^{13}C NMR and HMBC data of **2.2** in $CDCl_3$ (400 MHz for ^{1}H, 100 MHz for ^{13}C).

Position	**δ_H (mult, *J* in Hz)**	**δ_C**	**HMBC**
1	-	130.7	-
2	7.25 (1H, d, *J* = 8.5 Hz)	127.4	C-3, C-4, C-7
3	6.78 (1H, d, *J* = 8.5 Hz)	115.7	C-1, C-3, C-4
4	5.20 (OH, brs)	154.7	-
5	6.78 (1H, d, *J* = 8.5 Hz)	115.7	C-1, C-3, C-4
6	7.25 (1H, d, *J* = 8.5 Hz)	127.4	C-3, C-4, C-7
7	6.28 (1H, d, *J* = 16.5 Hz)	125.6	C-1, C-2, C-6, C-8, C-9, C-12, C-13
8	6.09 (1H, d, *J* = 16.5 Hz)	136.8	C-1, C-2, C-6, C-7, C-10, C-12, C-13
9	-	39.3	-
10	5.92 (1H, dd, *J* = 17.5, 10.5 Hz)	147.1	C-8, C-9, C-12, C-13,
11	4.95 (2H, m)	110.9	C-9, C-10
12-CH_3	1.19 (3H, s)	27.1	C-7, C-8, C-9, C-10, C-11
13-CH_3	1.19 (3H, s)	27.1	C-7, C-8, C-9, C-10, C-11

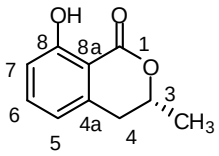


Antidesmol (2.1): colorless oil, ^{1}H NMR ($CDCl_3$); δ_H 1.19 (6H, s, CH_3-12,13), 3.90 (3H, s, OCH_3-3), 4.98 (2H, m, H-11), 5.61 (1H, br s, OH-4), 5.90 (1H, dd, *J* = 17.6, 10.4 Hz, H-10), 6.05 (1H, d, *J* = 16.4 Hz, H-8), 6.25 (1H, d, *J* = 16.4 Hz, H-7), 6.84 (1H , d, *J* = 8.0 Hz, H-5), 6.87 (1H, dd, *J* = 8.0, 1.6 Hz, H-6), 6.89 (1H, d, *J* = 1.6 Hz, H-2) ; ^{13}C NMR ($CDCl_3$): δ_C 27.0 (C-12,13), 39.3 (C-9), 55.8 (C- OCH_3), 108.1 (C-2), 110.8 (C-

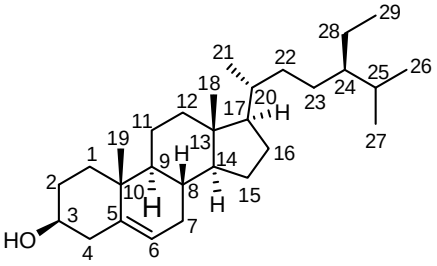
11), 114.3 (C-5), 119.6 (C-6), 126.1 (C-7), 130.4 (C-1), 136.6 (C-8), 144.9 (C-4), 146.5 (C-3), 147.1 (C-10) [50].



Corylifolin (2.2): white solid: mp 68-74 °C, ^{1}H NMR ($CDCl_3$); δ_H 1.19 (6H, s, CH_3-12,13), 4.95 (2H, m, H-11), 5.20 (1H, brs, OH-4), 5.92 (1H, dd, *J* = 17.5, 10.5 Hz, H-10), 6.09 (1H ,d, *J* = 16.5 Hz, H-8), 6.28 (1H, d, *J* = 16.5 Hz, H-7), 6.78 (2H , d, *J* = 8.5 Hz, H-3,5), 7.25 (2H, d, *J* = 8.5 Hz, H-2,6); ^{13}C NMR ($CDCl_3$): δ_C 27.1 (C-12,13), 39.3 (C-9), 110.9 (C-11), 115.7 (C-3,5), 125.6 (C-7), 127.4 (C-2,6), 130.7 (C-1),136.8 (C-8) 147.1 (C-10), 154.7 (C-4); HRMS (EI) *m*/z calcd for $C_{13}H_{16}ONa$ 211.1099, found 211.1126 [51].

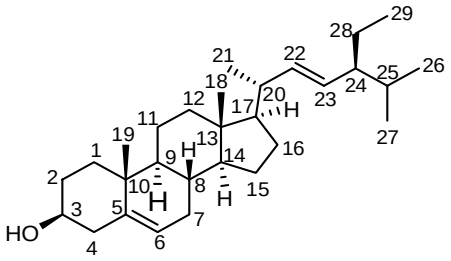


Mellein (2.3) : crystalline solid, ^{1}H NMR ($CDCl_3$); δ_H 1.53 (3H, d, *J* = 6.1 Hz, CH_3-3), 2.92 (2H, m, H-4), 4.72 (1H, m, H-3), 6.70 (1H, dd, *J* = 0.8, 7.4 Hz, H-7), 6.88 (1H, dd, *J* = 0.3, 8.4 Hz, H-5), 7.40 (1H, dd, *J* = 7.4, 8.4 Hz, H-5); ^{13}C NMR ($CDCl_3$): δ_C 21.1 (C, CH_3-3), 34.9 (C-4), 76.5 (C-3), 108.6 (C-8a), 116.6 (C-5), 118.3 (C-7), 136.7 (C-6), 139.8 (C-4a), 170.4 (C-1) [52].

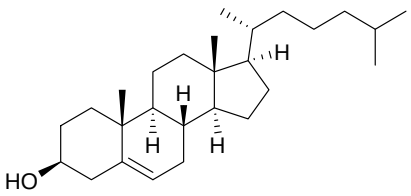


***β*-Sitosterol (2.4)**: colorless needles, ^{1}H NMR ($CDCl_3$) δ_H 0.72 (3H, s, CH_3-18), 0.85 (3H, d, *J* = 6.7 Hz, CH_3-27), 0.87 (3H, d, *J* = 6.7 Hz, CH_3-26), 0.89 (3H, t, *J* = 7.4 Hz, CH_3-29), 0.96 (3H, d, *J* = 6.5 Hz, CH_3-21), 1.05 (3H, s, CH_3-19), 5.39 (1H, m, H-6); ^{13}C-

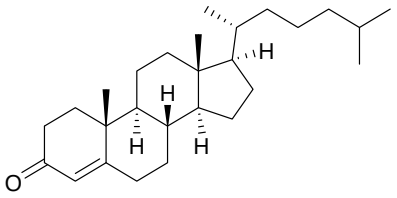
NMR ($CDCl_3$): δ_C 12.3 (C-29), 12.4 (C-18), 19.2 (C-21), 19.5 (C-27), 19.8 (C-19), 20.2 (C-26), 21.5 (C-11), 23.3 (C-28), 24.7 (C-15), 26.5 (C-23), 28.7 (C-16), 29.6 (C-25), 32.1 (C-7), 32.3 (C-2,8), 34.4 (C-22), 36.6 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2 (C-12), 42.8 (C-4,13), 46.2 (C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 72.2 (C-3), 122.1 (C-6), 141.2 (C-5) [20].



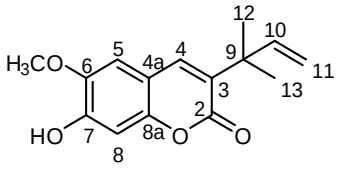
Stigmasterol (2.5): colorless needles, ^{1}H NMR ($CDCl_3$) δ_H 0.72 (3H, s, CH_3-18), 0.85 (3H, d, J = 6.7 Hz, CH_3-27), 0.87 (3H, d, J = 6.7 Hz, CH_3-26), 0.89 (3H, t, J = 7.4 Hz, CH_3-29), 0.96 (3H, d, J = 6.5 Hz, CH_3-21), 1.05 (3H, s, CH_3-19), 3.56 (1H, m, H-3),5.01 (1H, m, H-22), 5.15 (1H, m, H-23) 5.39 (1H, m, H-6); ^{13}C-NMR ($CDCl_3$): δ_C 12.3 (C-29), 12.4 (C-18), 19.2 (C-21), 19.5 (C-27), 19.8 (C-19), 20.2 (C-26), 21.4 (C-23) 21.5 (C-11), 23.3 (C-28), 24.7 (C-15), 28.7 (C-16), 29.6 (C-25), 32.1 (C-7), 32.3 (C-2,8), 36.6 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2 (C-12), 40.7 (C-22), 42.8 (C-4,13), 46.2 (C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 72.2 (C-3), 122.1 (C-6), 141.2 (C-5) [20].



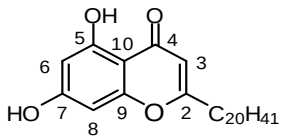
5-Cholesten-3β-ol (2.6): was characterized as 5-cholesten-3β-ol by comparison of the physical and spectral data with the literature [53].



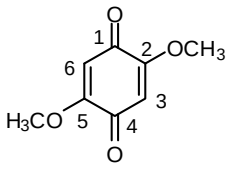
4-Choesten-3-one (2.7): was characterized as 4-choesten-3-one by comparison of the physical and spectral data with the literature [54].



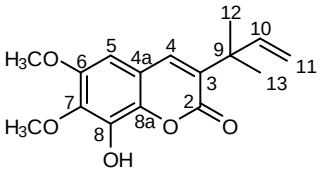
3-(1,1-Dimethylallyl)-scopoletin (2.8): Colorless needle, ^{1}H NMR ($CDCl_3$); δ_H 1.48 (6H, s, CH_3-12,13), 3.94 (3H, s, OCH_3-3), 5.09 (2H, m, H-11), 6.04 (1H, brs, OH-4), 6.18 (1H, dd, *J* = 17.2, 10.8 Hz, H-10), 6.84 (1H , s, H-5), 7.49 (1H, s, H-4) ; ^{13}C NMR ($CDCl_3$): δ_C 26.1 (C-12,13), 40.4 (C-9), 56.4 (C-OCH_3), 102.4 (C-8), 107.5 (C-5), 111.9 (C-4a), 112.1 (C-11), 132.0 (C-3), 137.7 (C-4), 143.8 (C-6), 145.6 (C-10), 147.0 (C-7), 148.8 (C-8a), 160.1 (C-2) [55].



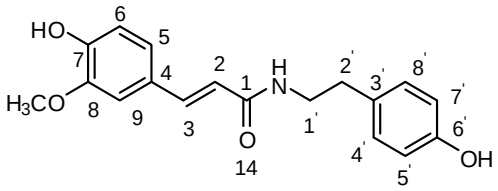
5**,7-Dihydroxy-2-eicosyl-chromone (2.9)**: white solid, ^{1}H NMR ($CDCl_3$); δ_H 2.57 (2H, t, *J* = 7.3 Hz, H-1'), 6.03 (1H, s, H-3), 6.28 (1H, d, *J* = 2 Hz, H-8), 6.34 (1H, d, *J* = 2 Hz, H-6), 6.52 (1H, brs, OH-7), 12.7 (1H, s, OH-5) ; ^{13}C NMR ($CDCl_3$): δ_C 34.2 (C-1'), 94.1 (C-8), 99.3 (C-6), 105.1 (C-10), 107.7 (C-3), 158.3 (C-5), 162.2 (C-9), 162.3 (C-7), 170.8 (C-2), 182.6 (C-4); HRMS (EI) *m*/z calcd for $C_{29}H_{46}O_4Na$ 481.3294, found 481.3268 [56].



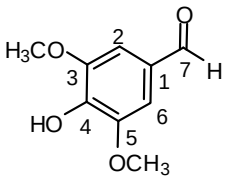
2,5-Dimethoxy-1,4-benzoquinone (2.10): yellow needles, ^{1}H NMR ($CDCl_3$); δ_H 3.75 (6H, s, OCH_3-3,6), 5.78 (2H, s, H-2,5); ^{13}C NMR ($CDCl_3$): δ_C 56.4 (C, OCH_3-3,6), 107.4 (C-2,5), 157.3 (C-3,6), 186.8 (C-1,4) [57].



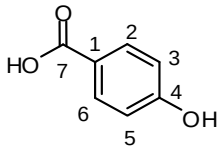
Barbatumol A (2.11): yellow solid, ^{1}H NMR ($CDCl_3$); δ_H 1.49 (6H, s, CH_3-12,13), 3.89 (3H, s, OCH_3-6), 3.98 (3H, s, OCH_3-7), 5.09 (2H, m, H-11), 5.95 (1H, brs, OH-8), 6.17 (1H, dd, *J* = 17.6, 10.8 Hz, H-10), 6.50 (1H , s, H-5), 7.50 (1H, s, H-4); ^{13}C NMR ($CDCl_3$): δ_C 26.1 (C-12,13), 40.6 (C-9), 56.2 (C-OCH_3-6), 61.3 (C- OCH_3-7), 100.0 (C-5), 112.4 (C-11), 114.5 (C-4a), 134.0 (C-3), 136.8 (C-8a), 138.0 (C-4,8), 141.3 (C-7), 145.3 (C-10), 149.5 (C-6), 159.0 (C-2) [55].



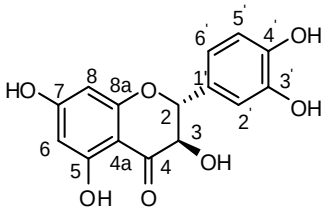
***N-trans*-Feruloyltyrramine (2.12)**: yellow oil; ^{1}H NMR (Acetone-d_6); δ_H 2.76 (2H, t, *J* = 7.7 Hz, H-2'), 3.51 (2H, t, *J* = 7.7 Hz, H-1'), 3.87 (3H, s, OCH_3-6) 6.53 (1H, d, *J* = 15.8 Hz, H-2), 6.70 (2H, d, *J* = 8.5 Hz, H-5',7'), 6.86 (1H, d, *J* = 8.1 Hz, H-8), 7.04 (1H, dd, *J* = 2.0, 8.0 Hz, H-9), 7.07 (2H, d, *J* = 8.5 Hz, H-4',8'), 7.16 (1H, d, *J* = 1.8 Hz, H-5), 7.48 (1H, d, *J* = 15.8 Hz, H-3); ^{13}C NMR ($CDCl_3$): δ_C 35.7 (C-2'), 41.9 (C-1'), 56.2 (OCH_3-6), 111.3 (C-5), 116.1 (C-8), 116.2 (C-5',7'), 119.9 (C-2), 122.5 (C-9), 128.2 (C-4), 130.5 (C-4',8'), 131.1 (C-3'), 140.5 (C-3), 148.6 (C-7), 149.1 (C-6), 156.7 (C-6'), 166.6 (C-1) [55].



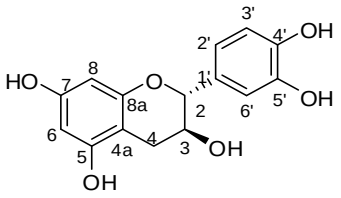
Syringic aldehyde (2.13): yellow solid, NMR ($CDCl_3$); ^{1}H NMR ($CDCl_3$); δ_H 3.92 (6H, s, OCH_3-3,5), 7.15 (2H, s, H-2, 6), 9.82 (1H, s, H-7); ^{13}C-NMR ($CDCl_3$): δ_C 56.5 (C-OCH_3-3,5), 106.7 (C-2, 6), 140.9 (C-4), 147.4 (C-3,5), 190.7 (C-7) [57].



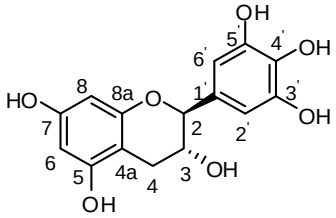
***p*-Hydroxybenzoic acid (2.14)**: yellow solid, ^{1}H NMR (Acetone-d_6); ^{1}H NMR ($CDCl_3$); δ_H 6.81 (2H, d, J = 8.76, H-3,5), 7.87 (2H, d, J = 8.80, H-2, 6); ^{13}C NMR (Acetone-d_6): δ_C 116.0 (C-3,5), 122.6 (C-1), 132.9 (C-2, 6), 163.3 (C-4), 170.1 (C-7) [58].



Taxifolin (2.15): yellow crystals, ^{1}H NMR (CD_3OD): δ_H 4.50 (1H, d, J = 11.0 Hz, H-3), 4.92 (1H, d, J = 11.0 Hz, H-2), 5.89 (1H, d, J = 2.0 Hz, H-8), 5.91 (1H, d, J = 2.0 Hz, H-6), 6.81 (1H, d, J = 8.0 Hz, H-5'), 6.86 (1H, dd, J = 2.0, 8.0 Hz, H-6'), 6.97 (1H, d, J = 2.0 Hz, H-2'); ^{13}C NMR (CD_3OD): δ_C 73.64 (C-3), 85.09 (C-2), 96.27 (C-8), 97.29 (C-6), 101.79 (C-4a), 115.85 (C-2'), 116.05 (C-5'), 120.88 (C-6'), 129.82 (C-1'), 146.28 (C-3'), 147.11 (C-4'), 164.27 (C-5), 164.47 (C-8a), 168.72 (C-7), 198.37 (C-4) [59].



(+)-Catechin (2.16): yellow powder, ^{1}H NMR (CD_3OD): δ_H 2.51 (1H, dd, *J* = 16.2, 8.0 Hz, H-4b), 2.85 (1 H, dd, *J* = 16.2, 5.4 Hz, H-4a), 3.99 (1H, ddd, *J* = 8.0, 7.6, 5.4 Hz, H-3), 4.56 (1H, d, *J* = 7.6 Hz, H-2), 5.88 (1H, d, *J* = 2.2 Hz, H-6), 5.95 (1H, d, *J* = 2.2 Hz, H-8), 6.72 (1H, dd, J = 8.2, 1.6 Hz, H-6′), 6.77 (1H, d, *J* = 8.2 Hz, H-5′), 6.84 (1H, d, *J* = 1.6 Hz, H-2′); ^{13}C NMR (CD_3OD): δ_C 28.6 (C-4), 69.0 (C-3), 83.0 (C-2), 95.8 (C-8), 96.6 (C-6), 100.1 (C-10), 115.5 (C-4′), 116.4 (C-2′), 120.3 (C-6′), 132.4 (C-1′), 146.4 (C-3',5′),157.1 (C-9), 157.7 (C-7), 157.9 (C-5) [24].



(-)-Gallocatechin (2.17): yellow solid , ^{1}H NMR (CD_3OD): δ_H 2.50 (1H, dd, *J* = 16.0, 8.0 Hz, H-4), 2.80 (1H, dd, *J* = 16.0, 5.0 Hz, H-4), 3.97 (1H, m, H-3), 4.53 (1H, d, *J* = 7.0 Hz, H-2), 5.86 (1H, d, *J* = 2.0 Hz, H-8), 5.92 (1H, d, *J* = 2.5 Hz, H-6), 6.40 (2H, s, H-2', 6'); ^{13}C NMR (CD3OD): δ_C 28.0 (C-4), 68.7 (C-3), 82.8 (C-2), 95.5 (C-8), 96.3 (C-6), 100.7 (C-4a), 107.2 (C-2', 6'), 131.5 (C-10), 134.0 (C-4'), 146.8 (C-3', 5'), 156.8 (C-8a), 157.5 (C-5), 157.8 (C-7) [60].

2.3.2 Bioassay activity of isolated compounds

2.3.2.1 Cytotoxicity against KB and HeLa cell lines of isolated compounds

The cytotoxicity towards HeLa and KB cell lines of all isolated compounds were evaluated using MTT colorimetric assay and the results were shown in Table 2.2

Table 2.2 *In vitro* cytotoxicity of compounds **2.1-2.17** against HeLa and KB cells.

Compound	IC_{50} (µg/mL)	
	KB	HeLa
Antidesmol (**2.1**)	>100	69
Corylifolin (**2.2**)	50.7	>100
Mellein (**2.3**)	24.4	51.5
A mixture of *β*-stitosterol (**2.4**) and stigmasterol (**2.5**)	>100	>100
5-Cholesten-3*β*-ol (**2.6**)	>100	>100
4-Cholesten-3-one (**2.7**)	>100	>100
3-(1,1-Dimethylallyl)-scopoletin (**2.8**)	17.6	32.1
5,7-Dihydroxy-2-eicosyl-chromone (**2.9**)	32.7	3.9
2,5-Dimethoxy-1,4-bezoquinone (**2.10**)	4.9	1.6
Barbatumol A (**2.11**)	34.8	36.6
N-trans-Feruloyltyrramine (**2.12**)	7.8	12.3
Syringic aldehyde (**2.13**)	>100	88.1
p-Hydroxybenzoic acid (**2.14**)	22.8	10.8
Taxifolin (**2.15**)	62.6	>100
(+)-Catechin (**2.16**)	88	36.6
(-)-Gallocatechin (**2.17**)	24.8	14.9
Adriamycin (standard)	0.018	0.018

From Table 2.2, all isolated compounds (**2.1-2.17**) were determined for their cytotoxicity against on KB and HeLa cells. Compounds **2.9** and **2.10** showed only good cytotoxicity against on HeLa cells (with IC_{50} values of 3.9 and 1.6 μg/mL) and compounds **2.12**, **2.14** and **2.17** showed only moderate cytotoxicity against on HeLa cells (with IC_{50} values of 12.3, 10.8 and 14.9 μg/mL), while compounds **2.10** and **2.12** showed only moderate cytotoxicity against on KB cells (with IC_{50} values of 4.9 and 7.8 μg/mL) and compounds **2.3**, **2.8**, **2.14** and **2.17** showed only weak cytotoxicity against on KB cells (with IC_{50} values of 24.4, 17.6, 22.8 and 24.8 μg/mL). In addition, other compounds with IC_{50} values more than 30.0 μg/mL could be regarded as inactive.

2.3.2.2 Anticholinesterase inhibitory activity

The anticholinesterase inhibitory activity of all isolated compounds were shown in Table 2.3

Table 2.3 AChE and BuChE inhibitory activity of compounds **2.1-2.17** from the roots of *A.acidum* Retz.

Compound	% inhibition	
	AChE	BuChE
Antidesmol (**2.1**)	NA	31.83
Corylifolin (**2.2**)	15.18	47.78
Mellein (**2.3**)	8.13	40.29
A mixture of *β*-stitosterol (**2.4**) and stigmasterol (**2.5**)	NA	NA
5-Cholesten-3*β*-ol (**2.6**)	NA	NA
4-Cholesten-3-one (**2.7**)	NA	NA
3-(1,1-Dimethylallyl)-scopoletin (**2.8**)	14.09	20.80
5,7-Dihydroxy-2-eicosyl-chromone (**2.9**)	NA	10.40
2,5-Dimethoxy-1,4-bezoquinone (**2.10**)	7.59	14.29
Barbatumol A (**2.11**)	NA	22.26
N-trans-Feruloyltyrramine (**2.12**)	6.50	43.62
Syringic aldehyde (**2.13**)	9.76	22.47
p-Hydroxybenzoic acid (**2.14**)	NA	7.84
Taxifolin (**2.15**)	15.18	25.24
(+)-Catechin (**2.16**)	4.34	21.36
(-)-Gallocatechin (**2.17**)	23.58	31.90
Eserine (standard)	99.33	99.10

% inhibition ≤ 40 = active NA = No Activity

% inhibition > 40 = inactive

According to the result in Table 2.2, all isolated compounds were tested for their AChE and BuChE inhibitory activities. Compounds **2.2**, **2.3** and **2.12** showed very low inhibitory activity toward only BuChE with percentage inhibitory activity values of 47.78, 40.29 and 43.62, respectively. Other compounds were regarded as inactive (% inhibition < 40.00).

2.4 Conclusion

In this phytochemistry investigation of the CH_2Cl_2 and MeOH crude extracts of the roots of *A.acidum* Retz. afforded a new synthetically known compound, corylifolin (**2.2**), along with sixteen compounds including antidesmol (**2.1**), mellein (**2.3**), a mixture of β-stitosterol (**2.4**) and stigmasterol (**2.5**), 5-cholesten-3β-ol (**2.6**, 30.5 mg) and 4-cholesten-3-one (**2.7**), 3-(1,1-dimethylallyl)-scopoletin (**2.8**), 5,7-Dihydroxy-2-eicosyl-chromone (**2.9**), 2,5-dimethoxy-1,4-bezoquinone (**2.10**), barbatumol A (**2.11**), *N-trans*-feruloyltyramine (**2.12**), syringic aldehyde (**2.13**), *p*-hydroxybenzoic acid (**2.14**), taxifolin (**2.15**), (+)-catechin (**2.16**) and (-)-gallocatechin (**2.17**).

All isolated compounds (**2.1**-**2.17**) were determined for their cytotoxicity on KB and HeLa cells. Compounds **2.9** and **2.10** showed only good cytotoxicity against on HeLa cells (with IC_{50} values of 3.9 and 1.6 μg/mL) and compounds **2.12**, **2.14**, and **2.17** showed only moderate cytotoxicity against on HeLa cells (with IC_{50} values of 12.3, 10.8 and 14.9 μg/mL), while compound **2.10** and **2.12** showed only moderate cytotoxicity against on KB cells (with IC_{50} values of 4.9 and 7.8 μg/mL) and compound **2.3**, **2.8**, **2.14** and **2.17** showed only weak cytotoxicity against on KB cells (with IC_{50} value of 24.4, 17.6, 22.8 and 24.8 μg/mL). In addition, other compounds with IC_{50} values more than 30.0 μg/mL could be regarded as inactive.

The evaluation for the AChE and BuChE inhibitory activity using Ellman colorimetric method indicated that compounds **2.2**, **2.3** and **2.12** showed very low inhibitory activity toward only BuChE with percentage inhibitory activity values of 47.78, 40.29 and 43.62, respectively. Other compounds were regarded as inactivity (% inhibition < 40.00).

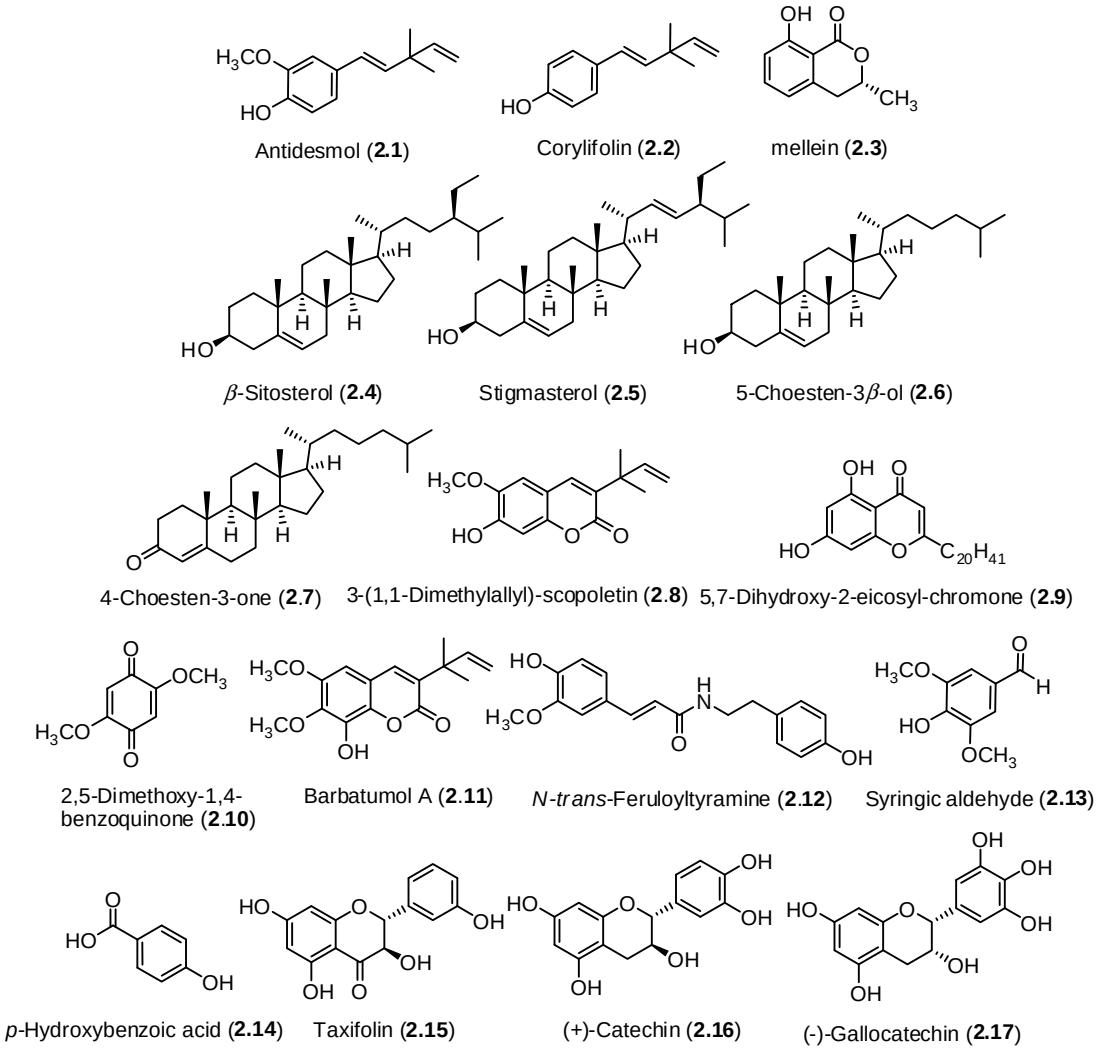


Figure 2.13 All of the isolated compounds (**1-17**) from the CH_2Cl_2 and MeOH crude extracts of *A. acidum* Retz. roots.

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APPENDIX

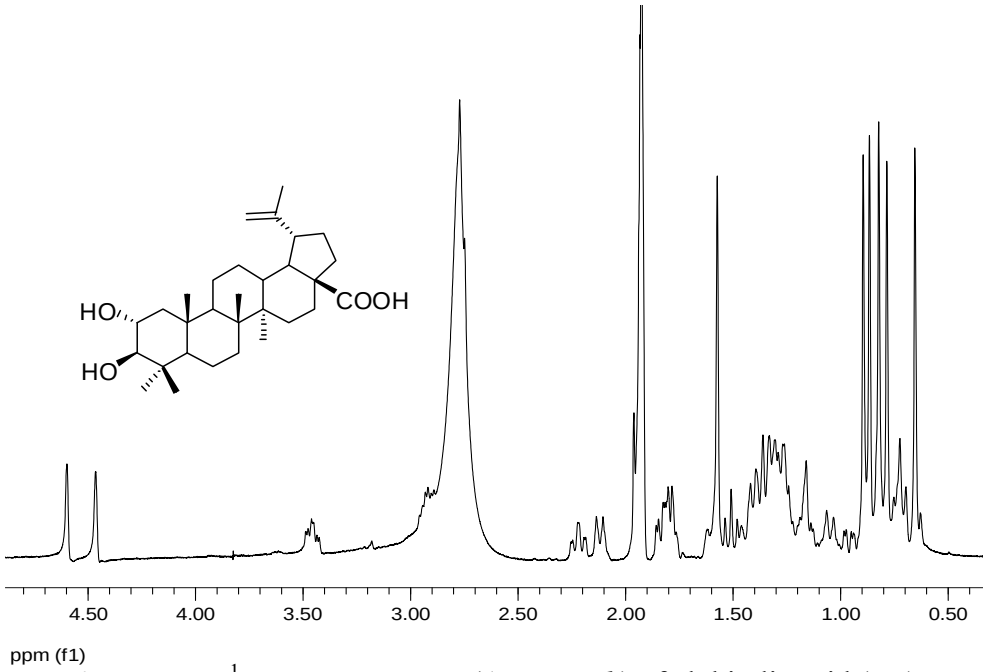

Figure A-1.1 ^{1}H NMR spectrum (Acetone-d_6) of alphitolic acid (**1.5**).

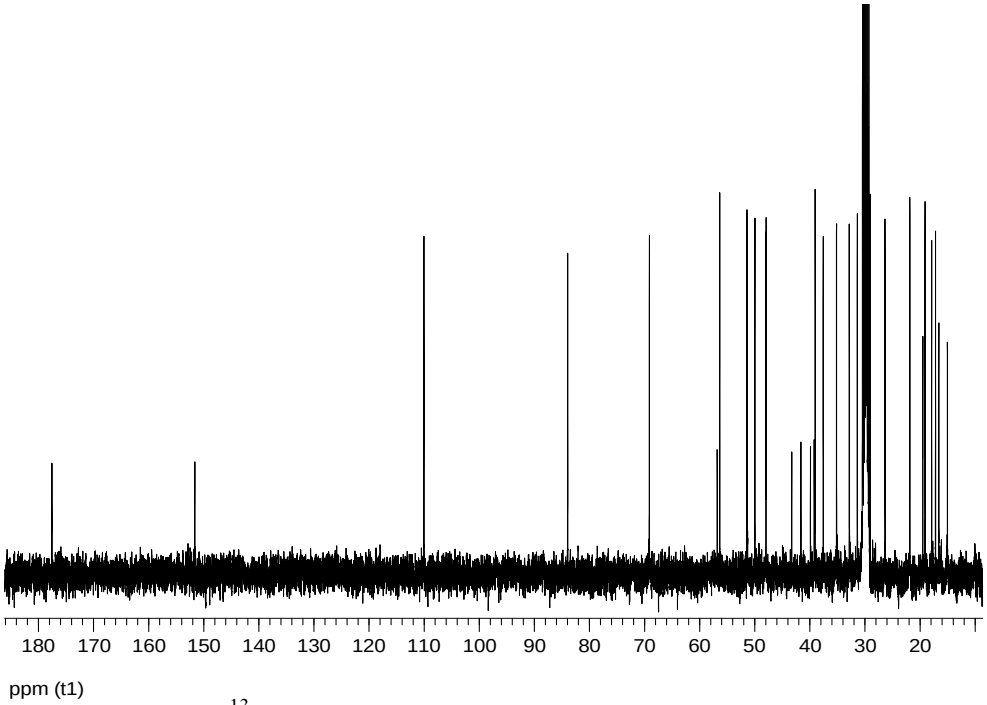

Figure A-1.2 ^{13}C NMR spectrum (Acetone-d_6) of alphitolic acid (**1.5**).

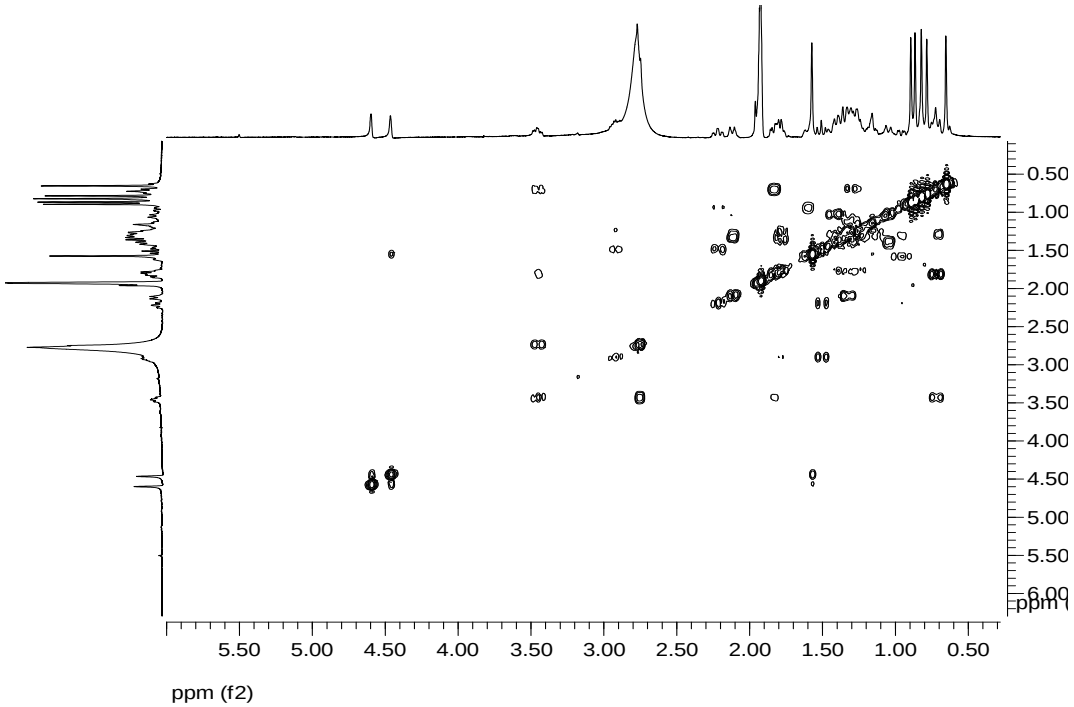

Figure A-1.3 COSY spectrum (Acetone-d_6) of alphitolic acid (**1.5**).

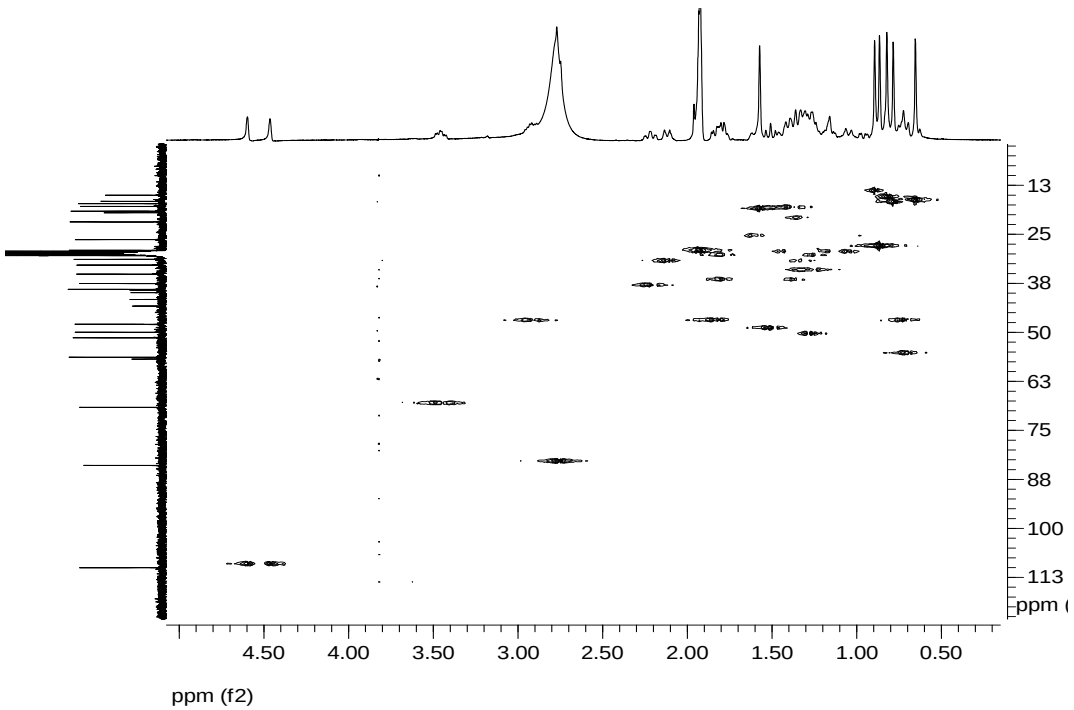

Figure A-1.4 HSQC spectrum (Acetone-d_6) of alphitolic acid (**1.5**).

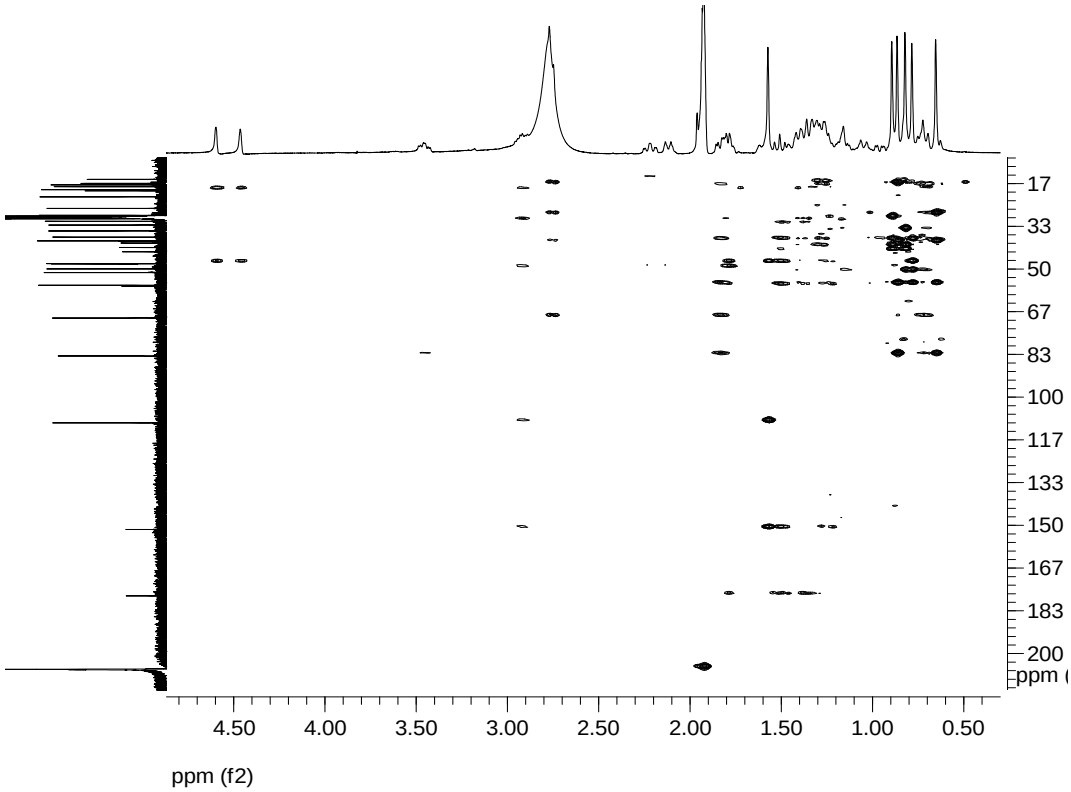

Figure A-1.5 HMBC spectrum (Acetone-d_6) of alphitolic acid (**1.5**).

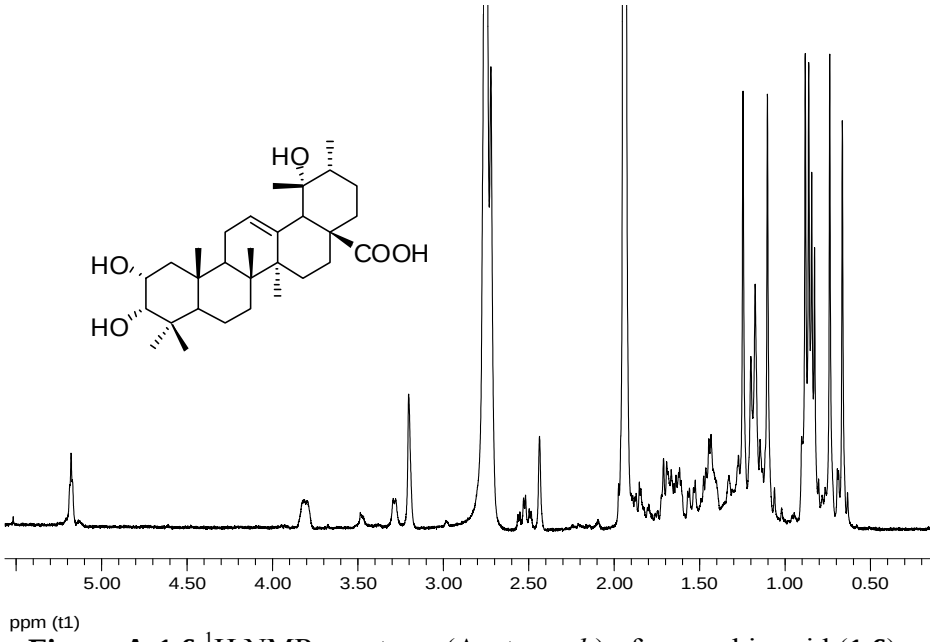

Figure A-1.6 ^{1}H NMR spectrum (Acetone-d_6) of euscaphic acid (**1.6**).

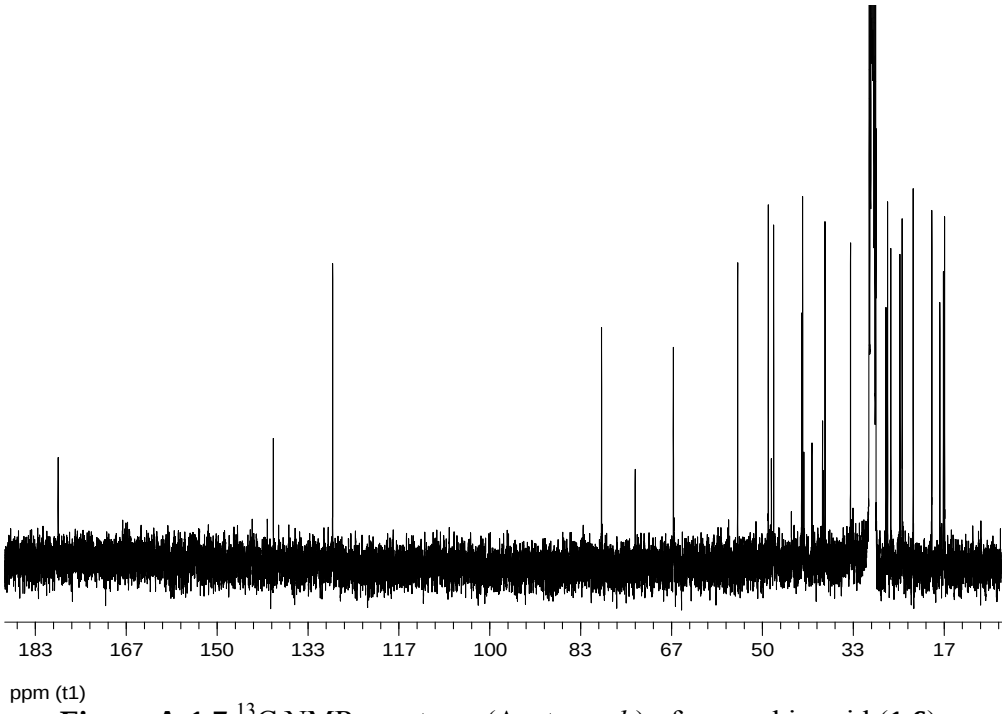

Figure A-1.7 ^{13}C NMR spectrum (Acetone-d_6) of euscaphic acid (**1.6**).

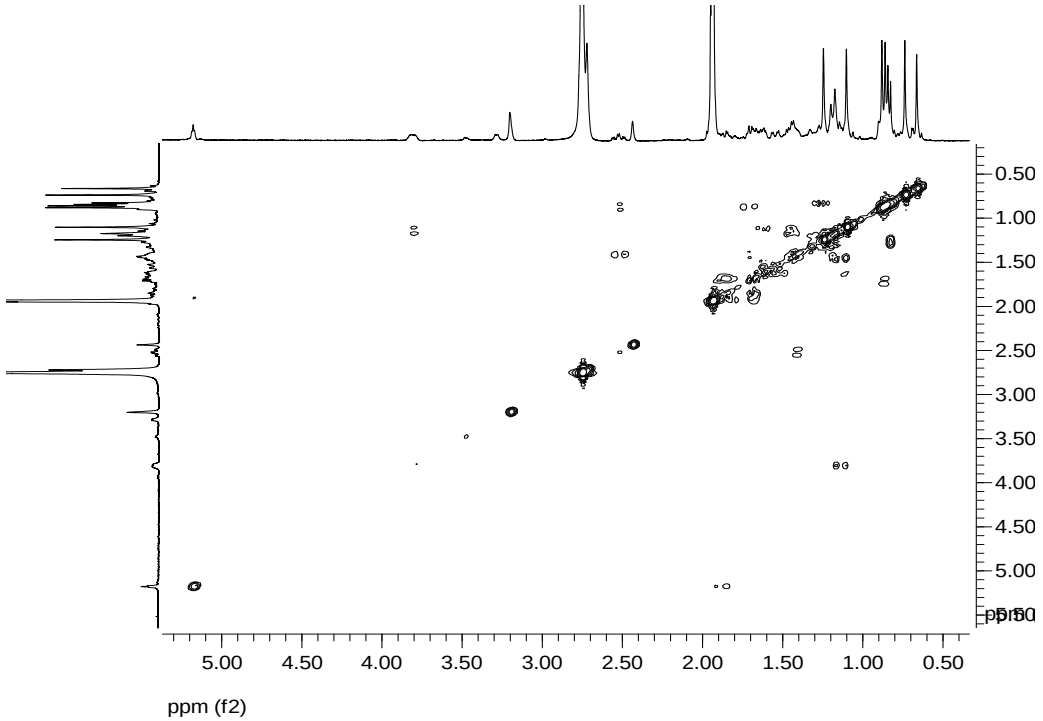

Figure A-1.8 COSY spectrum (Acetone-d_6) of euscaphic acid (**1.6**).

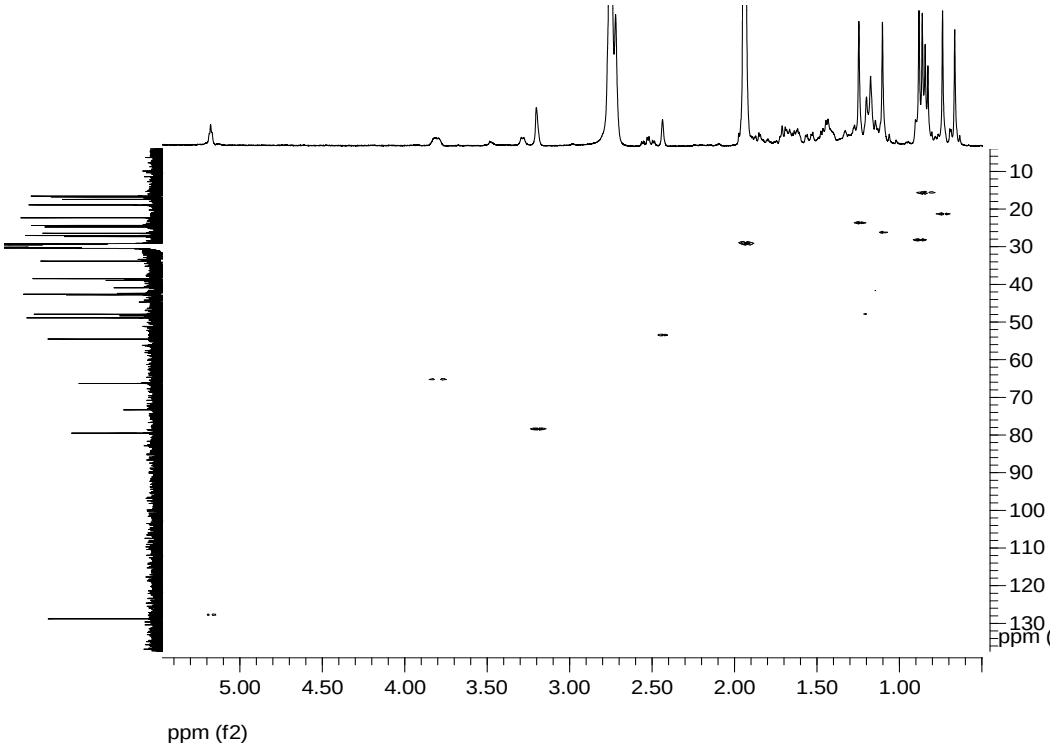

Figure A-1.9 HSQC spectrum (Acetone-d_6) of euscaphic acid (**1.6**).

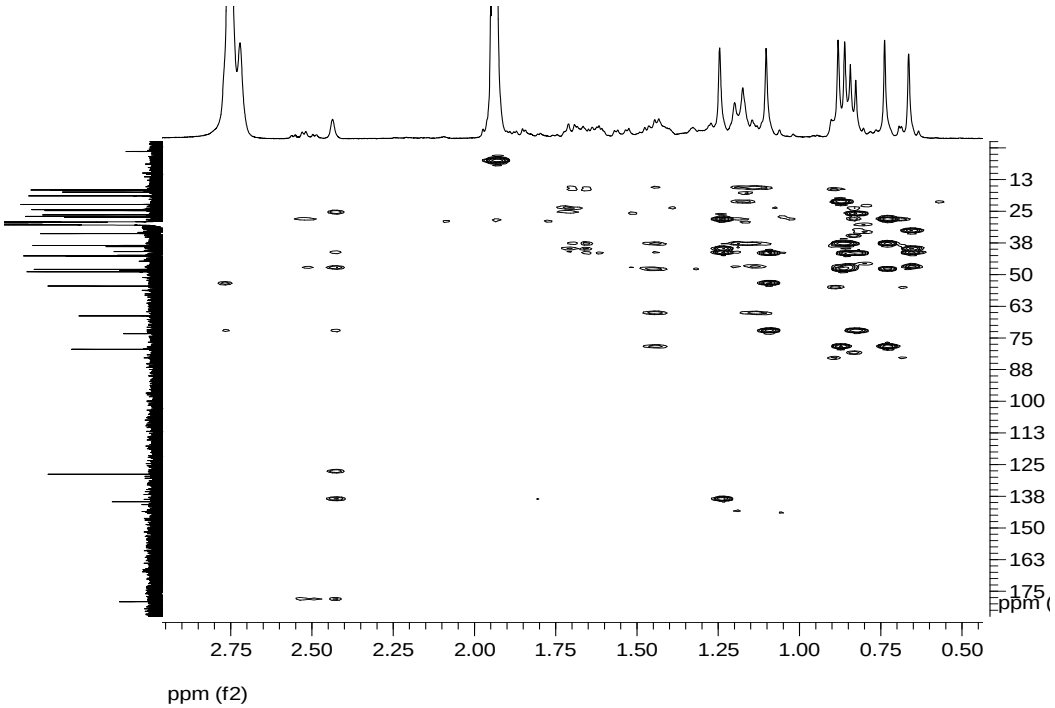

Figure A-1.10 HMBC spectrum (Acetone-d_6) of euscaphic acid (**1.6**).

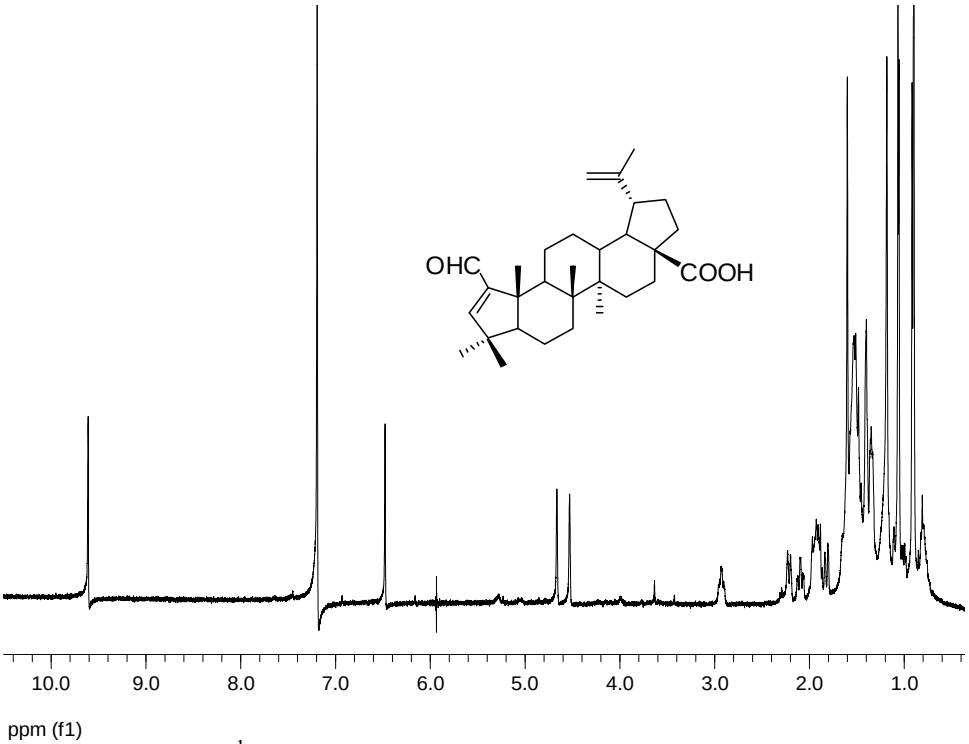

Figure A-1.11 ^{1}H NMR spectrum ($CDCl_3$) of zizyberenalic acid (**1.7**).

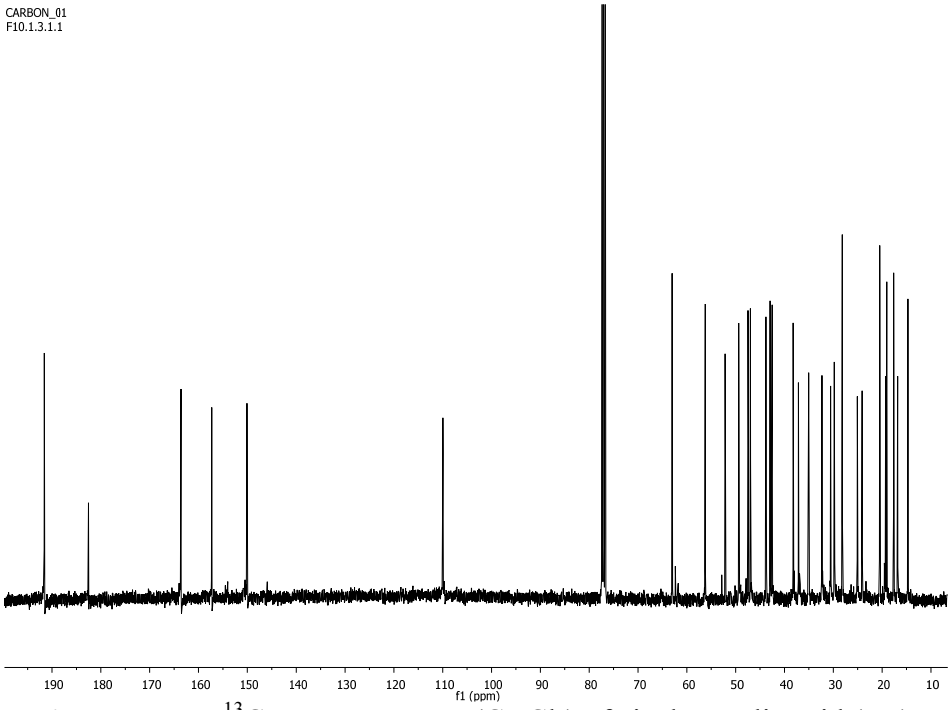

Figure A-1.12 ^{13}C NMR spectrum ($CDCl_3$) of zizyberenalic acid (**1.7**).

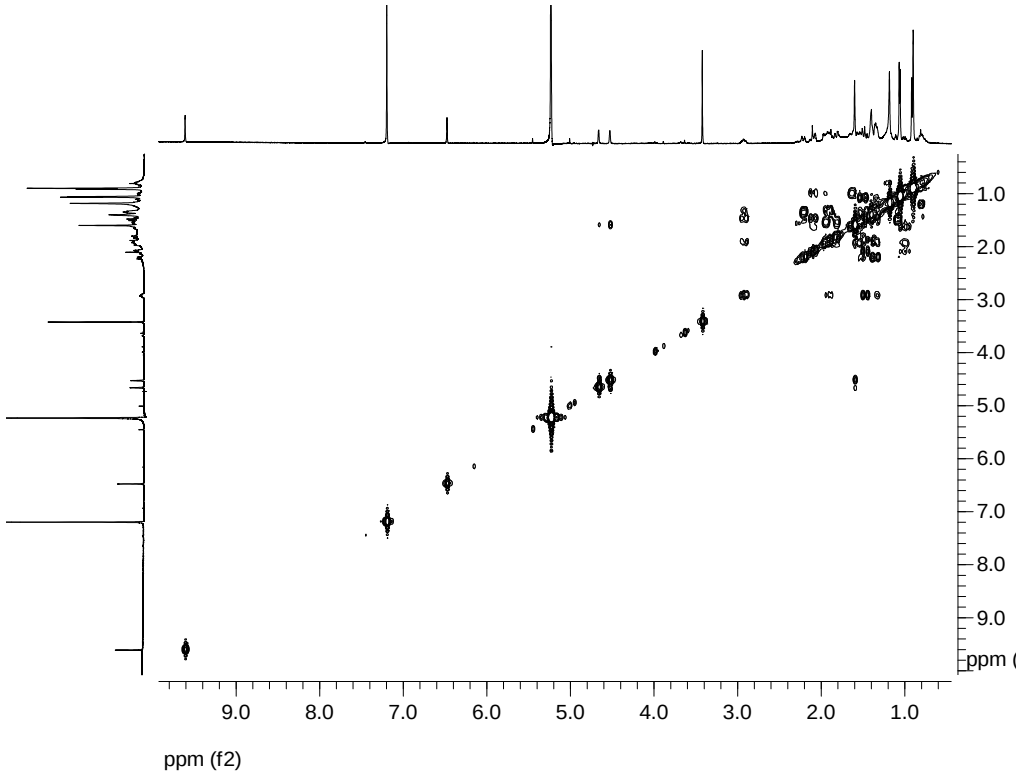

Figure A-1.13 COSY spectrum ($CDCl_3$) of zizyberenalic acid (**1.7**).

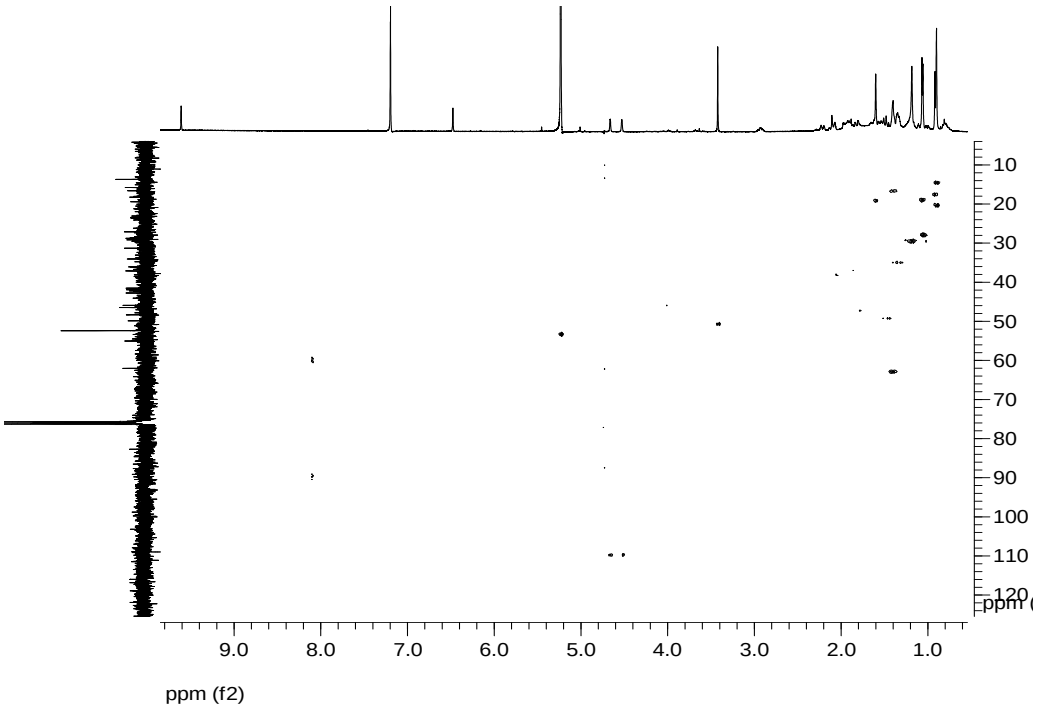

Figure A-1.14 HSQC spectrum ($CDCl_3$) of zizyberenalic acid (**1.7**).

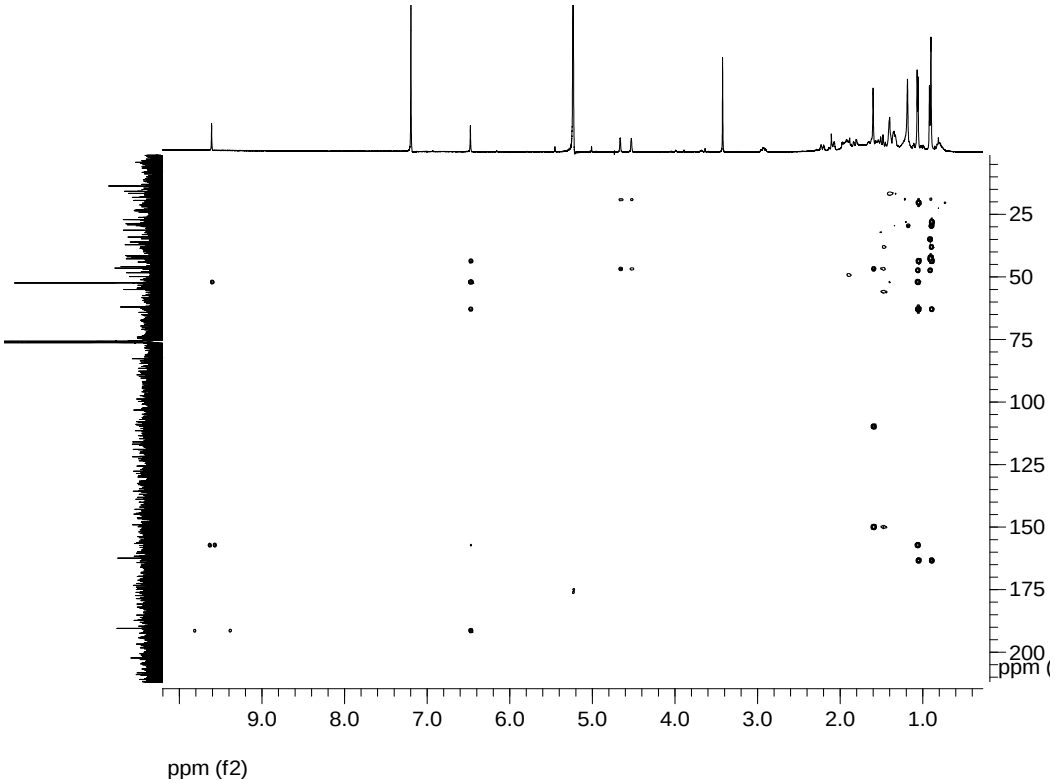

Figure A-1.15 HMBC spectrum ($CDCl_3$) of zizyberenalic acid (**1.7**).

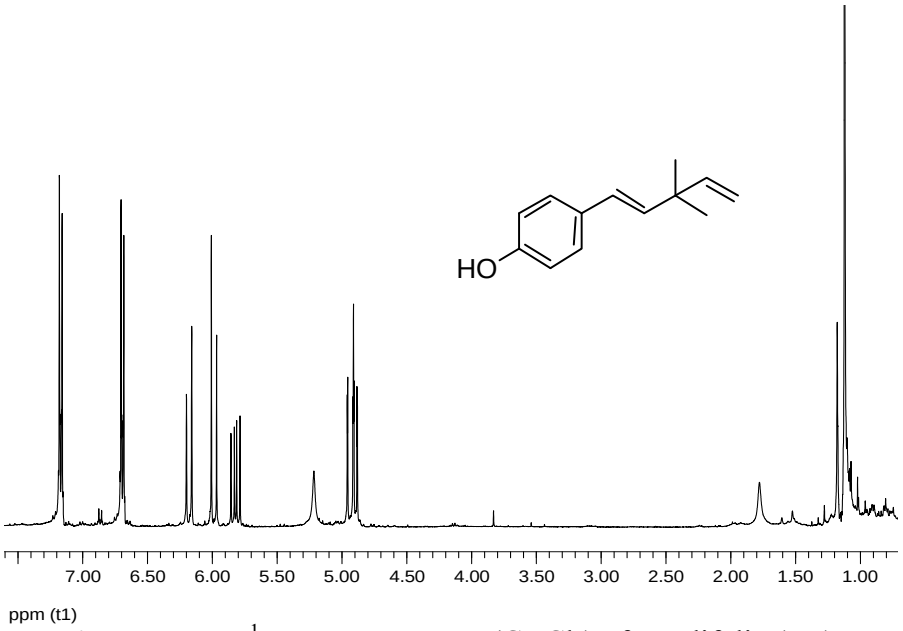

Figure A-1.16 ^1H NMR spectrum ($CDCl_3$) of corylifolin (**2.2**).

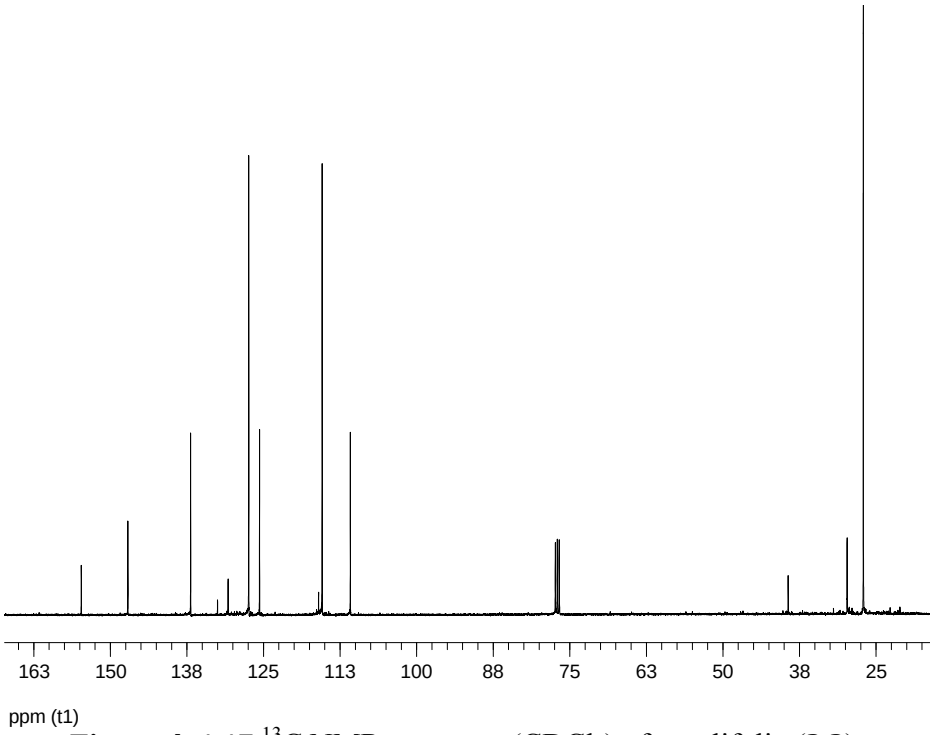

Figure A-1.17 ^{13}C NMR spectrum ($CDCl_3$) of corylifolin (**2.2**).

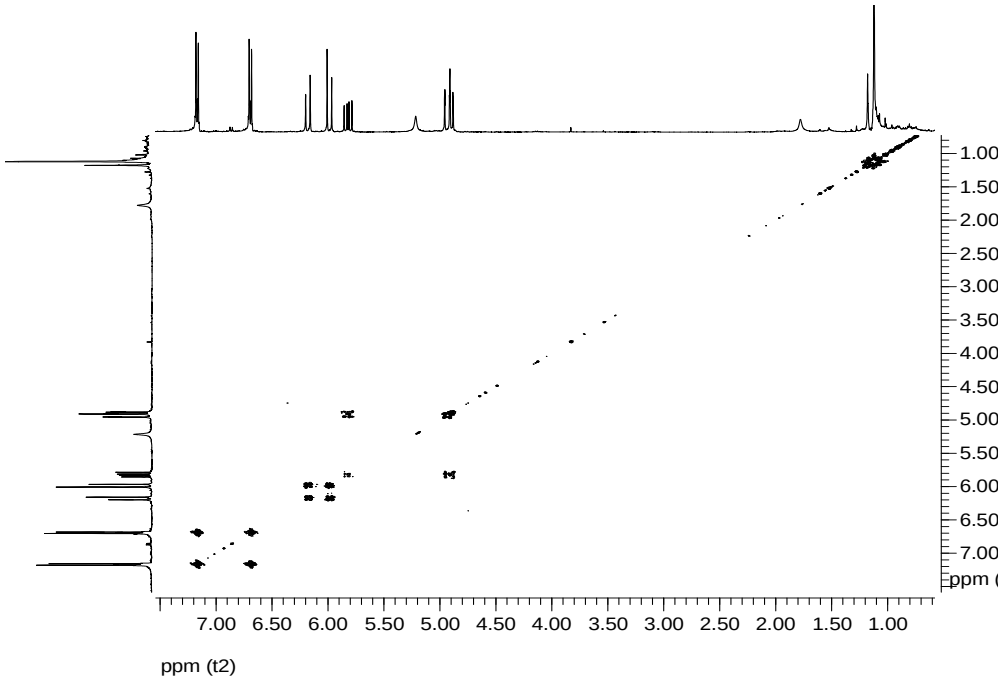

Figure A-1.18 COSY spectrum ($CDCl_3$) of corylifolin (**2.2**).

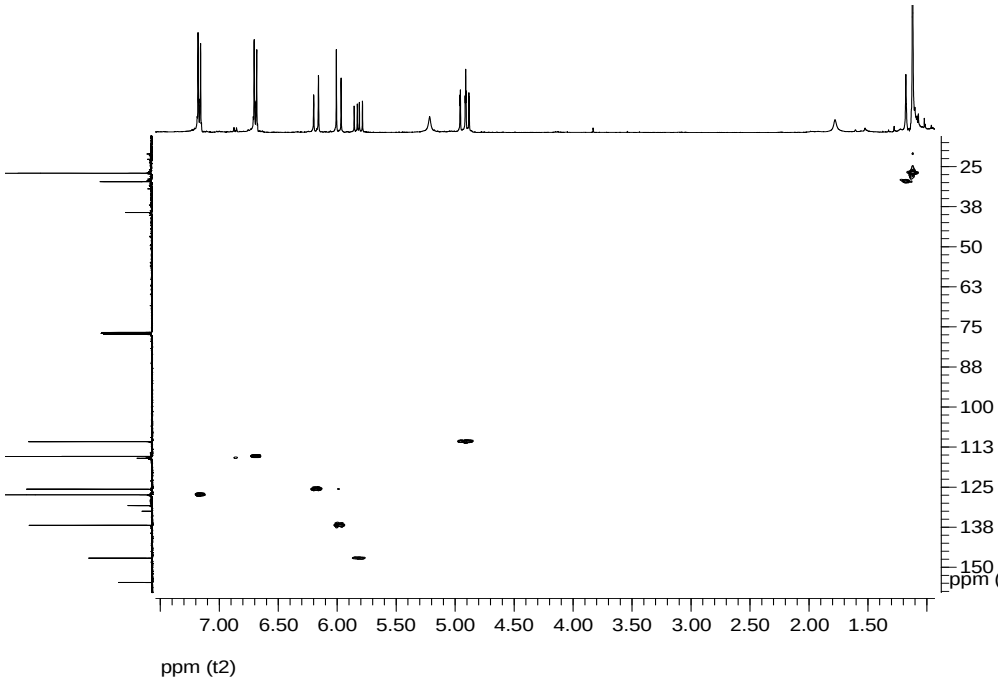

Figure A-1.19 HSQC spectrum ($CDCl_3$) of corylifolin (**2.2**).

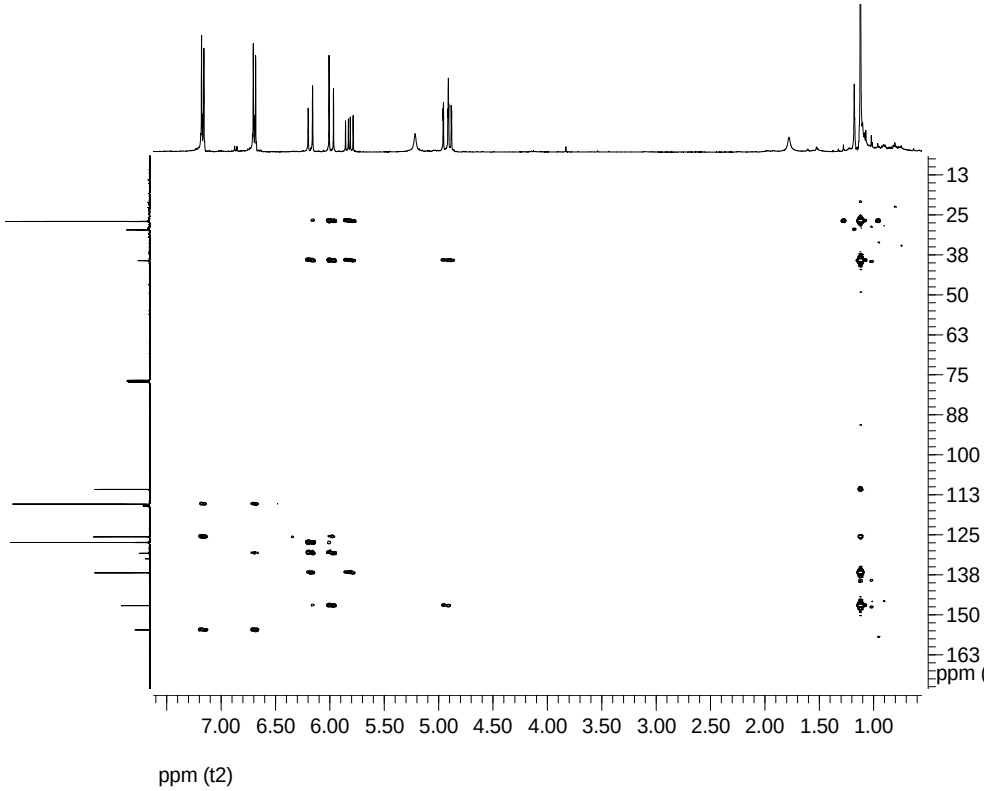

Figure A-1.20 HMBC spectrum ($CDCl_3$) of corylifolin (**2.2**).

VITA

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